Genetic and morphological impacts of a repeated fire regime on *Podarcis guadarramae*

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Sumário

Os fogos desempenham um papel importante na evolução e funcionamento de vários ecossistemas. Contudo, o aumento da densidade e distribuição da população humana levaram à modificação do regime natural de fogos. A rápida alteração deste regime devido ao aumento do número fogos e extensão das áreas ardidas torna importante o estudo do impacto dos fogos na biodiversidade. O estudo do impacto do fogo tem sido moderadamente estudado ao nível demográfico e funcional, porém o nível genético e morfológico é negligenciado. Este trabalho teve como objectivo avaliar o efeito do regime de fogos repetidos na diversidade e estrutura genética, bem como na morfologia de populações de *Podarcis guadarramae* no noroeste de Portugal. O esquema de amostragem foi composto por cinco localidades nas quais foram analisadas duas populações em áreas com diferentes condições: não-queimada e queimada. As áreas amostradas formavam um gradiente de histórias de fogo, isto é, com diferente número de fogos e tempo ocorrido desde o último fogo. Foram usados oito microssatélites para examinar o modo como o fogo afecta a diversidade genética, diferenciação e estrutura genética das populações. Ao nível da morfologia testamos a estabilidade do desenvolvimento através da medição do grau de assimetria fluctuante em três caracteres merísticos, dois funcionais (com importância biológica) e um não-funcional. As diversas análises apontam para um impacto positivo do fogo ao nível da variação genética, existindo um aumento dessa variação com o número de fogos; e, pelo contrário, uma diminuição com o tempo desde o último fogo. Foi detectada pouca diferenciação bem como ausência de estrutura genética entre as populações com diferentes condições. Os altos níveis de diversidade das populações de áreas queimadas podem ser causados pela sobrevivência e elevada imigração de indivíduos de zonas fora do perímetro do fogo, o que suporta a dinâmica populacional desta espécie. As populações de *P. guadarramae* de áreas queimadas mostraram estar sob desenvolvimento instável devido ao maior nível de assimetria quando comparado com as populações de áreas não-queimadas. Este resultado é, provavelmente, o reflexo do stress ambiental (isto é, maior temperatura e menor humidade) imposto pela simplificação da vegetação após fogos. Ainda assim, o nível de assimetria mostrou-se diferente consoante o caracter, cuja assimetria nos caracteres funcionais era menor que no caracter não-funcional. Uma possível consequência do maior grau de assimetria é a diminuição do desempenho do indivíduo, tal como a capacidade locomotora, que em troca pode aumentar a exposição a predadores. Este trabalho mostrou resultados mistos dependendo dos aspectos estudados em populações de *P. guadarramae* em resposta aos fogos. Isto destaca a importância de fazer uma análise multidisciplinar para estender o conhecimento sobre os efeitos dos fogos em populações naturais.

Palavras-chave: fogos, répteis, diversidade genética, estrutura genética, estabilidade desenvolvimento, assimetria, *Podarcis guadarramae*

Abstract

Wildfires are a crucial element that shapes the evolution and functioning of many world's ecosystems. However, the increase of human population density and distribution intensified and changed typical fire regimes. The rapid alteration of natural fire regimes by increasing the number of fires and extension of burnt areas urges the study of the impacts of wildfires on biodiversity. Despite the fact that the impacts of fires have been reasonably studied at demographic and functional levels, its impact on genetics and morphology of populations is underlooked and constitute a major knowledge gap. The objective of this work was to assess how the repeated fire regime affected the genetic diversity and structure, and also the morphology of *Podarcis guadarramae* populations in northerwestern Portugal. A paired sampling scheme was conducted in five replicate locations that were composed by two populations located in areas with different conditions: unburnt and burnt. The areas sampled differed in the number of fires and time since fire in order to have a gradient of populations with distinct fire-histories. Eight microsatellite loci were used to examine how the patterns of genetic diversity, differentiation and structure of populations were affected by fires. At the morphological level we tested for developmental stability by measuring the degree of fluctuating asymmetry of three meristic traits: supracilliar granules, femoral pores and the subdigital lamellae of the fourth toe of the hindlimb. Multiple analyses consistently pointed to a positive impact of fires on the genetic diversity of this lizard with a general trend of increasing diversity with the number of fires and time since the last fire. Little differentiation and no genetic structure were detected between populations from contrasting conditions. High diversity levels in populations of burnt areas may be a consequence of survival and high immigration from outside the fire boundary, supporting the metapopulation dynamic of *P. guadarramae*. Populations from burnt areas showed to be under developmental instability due to higher levels of asymmetry when compared with populations from unburnt areas. This result probably reflects environmental stress imposed by vegetation simplification after fire (this is, higher temperature and lower humidity). Still, the asymmetry was trait-specific in which femoral pores and subdigital lamellae showed less asymmetry levels. One possible consequence of higher asymmetry level is lower fitness, such as locomotor performance that in turn can increase exposure to predators. Our work showed mixed results regarding the different aspects studied in lizard populations as a response to wildfires. This fact highlights the importance of performing an integrative analysis to extend our understanding on the effects of wildfires on natural populations.

Keywords: fires, reptiles, genetic diversity, genetic structure, developmental stability, asymmetry, *Podarcis guadarramae*

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List of Abbreviations

AI – Asymmetry index

AMOVA – Analysis of molecular variance

ANOVA – Analysis of variance

A_R – Allelic richness

Bp – base pairs

BU - Burnt

DA – Directional asymmetry

DNA - Deoxyribonucleic acid

D_{ST} – Nei's standard genetic distance

FA – Fluctuating asymmetry

F_{CT} – Variance among groups relative to the total variance

F_{IS} – Inbreeding coefficient

FP – Femoral pores

F_{SC} – Variance among subpopulations within groups

F_{ST} – Variance among subpopulations relative to the total variance (i.e. Fixation index)

H_E – Expected heterozygosity

H_O – Observed heterozygosity

Hum - Humidity

HWE – Hardy-Weinberg equilibrium

IAM – Infinite allele model

IMH - Individual multilocus heterozygosity

L - Left

LD – Linkage desiquilibrium

m – Migration rate

M – Multi-scaled migration rate

MCMC - Markov chain Monte Carlo

N_A – Mean number of alleles

N_E – Effective population size

NE - Northeast

NW - Northwestern

P_A – Private alleles

PCoA – Principal coordinate analysis

PCR – Polymerase chain reaction

PNPG – Parque Nacional da Peneda-Gerês

R – Right

SCG – Supracilliar granules

SDL – Subdigital lamellae

SMM – Stepwise mutation model

SS – Sum of squares

STR – Short tandem repeats

T - Temperature

TPM – Two-phase model

TSLF – Time since the last fire

UN - Unburnt

UV – ultraviolet

VC – Variance component

1. Introduction

Content

1.1. Shaping ecosystems: natural disturbances

Disturbances, both human-induced and natural, act to maintain and promote ecosystem structure and function across a wide range of scales (e.g. Attiwill, 1994; Ackerly, 2004; Hughes *et al.*, 2005; Spies *et al.*, 2006). Disturbance can be defined as "any relatively discrete event in time that disrupts ecosystems, community, or population structure and changes resources, substrate availability, or the physical environment" (Pickett and White, 1985). In origin, disturbances can be abiotic (e.g. hurricanes, drought, etc.), biotic (e.g. invasive species, grazing, etc.), or the combination of both (e.g. fires are dependent from abiotic conditions as well as the adequate fuel which is biotic). Because disturbances alter the state and the trajectory of an ecosystem – interruption of ecological succession, they are key drivers of spatial and temporal heterogeneity of many natural communities (Turner, 2010). In turn, landscape heterogeneity (i.e. mosaic patches in different successional states) may enhance or retard the spatial spread of the disturbance (Turner, 1989). Yet, the relative importance of different disturbances varies among systems and can even vary through time in the same system. The spatial and temporal dynamics of disturbance over a large period of time is called a disturbance regime and include the spatial distribution of disturbances, its frequency and return interval, and also its size, intensity and severity.

Many natural disturbances are inherent in the internal dynamics of ecosystems, and often set the timing of ecosystem renewal processes fundamental for maintaining resilience in ecosystems (Holling *et al.*, 1995). There are biomes throughout the world that are naturally disturbance-prone adapted, for instance the Mediterranean vegetation which is well adapted to a repeated fire regime (Naveh, 1975; Pausas and Verdú, 2005), or the case of plant adaptation to flooding stress (Jackson and Colmer, 2005). Most likely, the disturbance response strategies are a balanced resistance-resilience trade-off (Miller and Chesson, 2009).

1.2. Fire as a worldwide disturbance

Wildfires have played a determining role in shaping the evolution and functioning of many ecosystems around the world (Bond and Keeley, 2005; Bond *et al.*, 2005; Bowman *et al.*, 2009). More than a local ecological disturbance, fire is a global ecosystem process (Bowman *et al.*, 2009), with approximately a third of the land mass experiencing intensive burning (Chuvieco *et al.*, 2008).

Fire, as a natural process, has been part of the Earth system since plants provided the fuels and oxygen necessary for flame, dated by the existence of charcoal

at about 420 million years (Pausas and Keeley, 2009). With the global climatic variations through geological time, fluctuation in the concentration of atmospheric oxygen was also an important determinant of fire activity (Scott and Glasspool, 2006). Fire has been an important selective factor for plant evolution and has shaped the development of some biomes (Bond *et al.*, 2005). Molecular phylogenies that were calibrated with fossil data could time the relationship between fire and the evolution of plant traits that enabled plants to survive and even promote fire (Bowman *et al.*, 2013). In fire-prone environments, adaptive traits such as resprouting, serotiny, germination triggered by heat-shock or smoke enable plants to adapt to a particular fire regime (Keeley *et al.*, 2011). Also, evolution of flammability implies selection for traits that increase the frequency or intensity of fire (Keeley *et al.*, 2011), since the expansion of the most fire-prone ecosystem, i.e. savannas, across Asia, Africa, and the Americas at about 7-8 million years ago coincide with a substantial pick in charcoal in marine sediments (Keeley and Rundel, 2005). Furthermore, it was shown that in the absence of fire savannas would be replaced by forest and therefore would not be so widely distributed (Bond *et al.*, 2005). This means that the balance between savannas and closed forest result from a balance between tree growth rates and fire frequency. Hence, large areas of Earth can switch between pyrophobic forests and pyrophytic vegetation mediated by vegetation, fire, and environment. However, shifts in fire regime and disruption of fire-vegetation interaction can lead to a rapid state change (Bowman *et al.*, 2013). The mastery of fire by hominids had the potential to cause such change.

Humans started to use fire since the Quaternary, although the routine domestic use of fire began around 50,000 to 100,000 years ago (Bar-Yosef, 2002). In the beginning of the Holocene, due to the development of sedentary agriculture-based societies, agricultural fires represented one of the major transformation of Earth at the time (Bowman *et al.*, 2013) and are in agreement with an increasing trend in vegetation fires since the start of the current interglacial period around 12,000 years ago (Power *et al.*, 2008). Nevertheless, there is evidence that climate variation has been a more influential driver of fire activity than burning by humans (Mooney *et al.*, 2011).

Until 1750, global biomass burning tracked mean global temperature, indicating the dominance of climate regulation of fire regimes (Marlon *et al.*, 2008). Over the past three centuries, human population density and distribution have increased exponentially which intensified the human influence of fire impacts (O'Connor *et al.*, 2011). Although climate continues to be an important factor in global fire regimes, in recent decades a shift from climate-controlled towards human-controlled fire regimes has been observed over the globe (Marlon *et al.*, 2008; Bowman *et al.*, 2013). Subsequent land cover conversion from forests and scrublands to agriculture uses, did biomass burning begin

to deviate from global mean temperature records (Marlon *et al.*, 2008). Humans are now shaping biomes with fire, such as boreal (Kasischke and Turetsky, 2006) and tropical forests (Nepstad *et al.*, 1999). On the contrary, in semi-arid temperate forests that were once prone to frequent natural fire (e.g. *Pinus ponderosa* in western US; Allen *et al.*, 2002), human-imposed fire suppression increased the landscape fuels which in turn increased the risk of intense wildfire and led to shifts in fire-regime from low- to high-intensity. Also, fire seasons are changing in response to climate change driven, in turn, by the release of greenhouse gases from the combustion of fossil fuels and by clearing and burning forests (Bowman *et al.*, 2009).

It is well recognized that contemporary patterns of fire activity are linked to climate, biology and human socioeconomic influences (O'Connor *et al.*, 2011; Bowman *et al.*, 2013; Hantson *et al.*, 2015). Across the globe, fire is controlled by the coincidence of three basic requirements: vegetative resources to burn, environmental conditions that promote combustion, and ignitions (Krawchuck *et al.*, 2009). While the physical process of combustion is theoretically simple, understanding the relative influence of biotic and abiotic controls on current altered fire regimes is blurred by the role of humans who are changing landscapes to be more or less flammable, as well as lighting and extinguishing fires (Chuvieco *et al.*, 2008; Krawchuck *et al.*, 2009). Owing to the recognition of the importance of fire and strong evidences that severe fire incidents have been increasing globally in recent years, a new concept was created – pyrogeography (Krawchuk *et al.*, 2009). It attempts to quantify observed variability in fire activity as a function of the complex interplay of environmental factors. This approach spans geographic scales from the local to the global, has an evolutionary frame, and thus has a geological dimension (Bowman *et al.*, 2013). Therefore, this discipline provides new perspectives on landscape fire management in a changing world, including its effects on biological conservation and global carbon cycle, and also linking fires to human health and cultures (Bowman *et al.*, 2013).

1.3. Fire-prone ecosystems: the Mediterranean Basin

Fire-dependent ecosystems now cover a large proportion of the global land surface and contain primarily fire-tolerant species adapted to a particular fire regime (Bowman *et al.*, 2009; Keeley *et al.*, 2011). A great example are the Mediterranean-type climate regions in the world: the Mediterranean Basin, California, central Chile, Cape Province in South Africa, and Western and South Australia; which stands as one of the classic example of convergent evolution (Keeley *et al.*, 2012). One major selective agent of this global

convergence is fire, which has been proved as an important factor affecting plant evolution in these regions (Keeley *et al.*, 2012). These landscapes share a similar climatic regime of a wet and mild temperature winter, alternating with a summer generally characterized by a combination of high temperatures and drought. This results in plant growth sufficient to produce high and continued fuel loads that are highly flammable during the summer drought, which makes these environments prone to high-intensity fires (Pausas, 2004; Keeley, 2012). Under these conditions, the frequency of wildfires tends to increase, being further enhanced by high wind speeds and heat waves also predominant in that season (Fernandes, 2013). The characteristic soil drought observed in these regions resulted in a significant expansion of suitable habitat for the evergreen sclerophyllous-leaved shrubs and woodlands that show typical fire regime adapted traits (Keeley *et al.*, 2012). Nonetheless, each region has unique characteristics relative to the range of plant responses to fire, due to different phylogenetic histories, as well as climatic and geological variation (Keeley, 2012; Ne'eman *et al.*, 2012); e.g. post-fire vegetative resprouting was selected in the Mediterranean Basin and California, while in the remaining Mediterranean regions post-fire seeding is more widespread (Keeley *et al.*, 2012).

In the Mediterranean Basin, fires are frequent since the late Quaternary (Carrión *et al.*, 2003), but the acquisition of adaptive mechanisms to persist and regenerate after recurrent fires dates back to earlier times (Naveh, 1975; Pausas and Verdú, 2005), possibly in the Pliocene (Jimenéz-Moreno *et al*., 2010). This, in turn, produced a structured biodiversity of plants as a function of different fire regimes (Verdú and Pausas, 2007). Landscapes of this region have been shaped by a several thousand-year history of human land use and disturbance (Naveh, 1975; Naveh, 1990; Pausas and Vallejo, 1999). The western part of the European Mediterranean Basin which includes the Iberian Peninsula, southern France and Sardinia has experienced a high frequency of vegetation fires in the last decades (Schmuck *et al.*, 2013). Interestingly, a major shift in the fire regime of this part of the basin over the past 130 years was identified around 1970 where the occurrence of fires after this period doubled, and the burned area increased by one order of magnitude (Pausas and Fernández-Muñoz, 2012). The main driver of this shift was the increase in fuel amount due to rural abandonment (Pausas and Fernández-Muñoz, 2012), but the period post-1970 was mostly correlated with climatic conditions such as summer meteorological conditions (Pereira *et al.*, 2005) and drought episodes (Pausas and Fernández-Muñoz, 2012).

Most Global and Regional Climate Models project an important warming in the Mediterranean Basin (Brands *et al.*, 2011, Ramos *et al.*, 2011; Frias *et al.*, 2012) leading to an increased fire activity (Batllori *et al.*, 2013; Bedia *et al.*, 2014). It is also expected

significant changes in precipitation regimes, mostly in monthly mean precipitation and intra-annual variability, characterized by less precipitation outside the winter season, as well as a tendency toward more extreme events (Giorgi and Lionello, 2008; Argüeso *et al.*, 2012). In western areas of Iberia, meteorology alone is more crucial on wildfire activity (Trigo *et al.*, 2013), and given the current climate change projections in the next century, Sousa *et al.* (2015) predicts that the mean burnt areas could be more than double by 2075. Departure from a particular fire regime can have devastating impacts on the sustainability of many ecosystem components (Pausas and Keeley, 2014).

1.3.1. Portugal: the most burnt country in Europe

Portugal is one of the countries in Europe with the highest amount of burnt land, and wildfire is considered one of the most important agents of landscape change in Portugal (Silva *et al.*, 2011). However, fire frequency and intensity have increased remarkably since the 1960s (Moreira *et al.* 2001; Pereira *et al.*, 2006). This increase was attributed to a combination of socioeconomic and environmental conditions in which the history of large-scale forest fires in Portugal is fairly recent (1950 onwards) and has become most evident since the beginning of the 1980s when the forestry authorities started to record fire occurrences in Portugal (Pereira *et al.*, 2011). On one hand, the cease of traditional land use practices (e.g. livestock grazing and wood harvesting) due to rural abandonment led to the natural process of ecological succession and subsequent scrubland encroachment (Moreira *et al.*, 2001). On the other hand, areas of marginally productive agriculture were converted to plantations of fire-prone tree species such as eucalypt and pine (Moreira *et al.*, 2011; Fernandes *et al.*, 2013). Moreover, the climate is warm temperate characterized by hot, dry summers and cool, wet winters; likewise, natural vegetation is typically evergreen, resistant to drought, and pyrophytic (Nunes *et al.*, 2005). In both cases (socioeconomic and environmental conditions) there is a higher accumulation of fuel which lead to a high risk of fire (Moreira *et al.*, 2009). However, many studies in this region have concluded that human-related factors were the most important factors that determines the spatial and temporal patterns of ignition (see Rego and Silva, 2014 and references therein). For example, Catry *et al.* (2009) concluded that human activities were the primary cause of wildfires since about 60% of the ignitions were linked to population density and close to roads.

Figure 1.1. Northern Portugal has the highest fire frequency in Europe due to its dominant fire-prone vegetation. Photo: AgênciaLusa.

In the last 30 years the average percentage of forest area burnt in Portugal is approximately 3% each year (JRC/EU, 2010), which is, by far, the highest fire incidence in Europe (Nunes *et al.*, 2005; Catry *et al.*, 2006; Oliveira *et al.*, 2012). In the period 1980-2010, over 3.2 million hectares burnt, which represents about 33% of the country's area (Rego and Silva, 2014); in the period 2003 and 2005, the burnt area reached its highest annual values since 1980 (750,932 hectares) (Martins *et al.*, 2012). Marques *et al.* (2011) used remote sense data from 1975 to 2007 and concluded that the area burnt in a single year ranged from 15,000 in 1977 to a catastrophic 440,000 hectares in 2003, with the largest fire covering 58,000 hectares in the year of 2003. Pereira *et al.* (2005) pointed out that this inter-annual variability of burnt area is largely determined by two different atmospheric factors: the amount of precipitation during spring (i.e. in the pre-fire season) and the occurrence of abnormally hot and dry atmospheric conditions through the summer season (i.e. throughout the fire season).

In Portugal, the northern part of the country has the highest fire frequency and with large fires that are responsible for the vast majority of the burned area in the country (Nunes *et al.*, 2005; Pereira *et al.*, 2006) due to the dominance of fire-prone tree species (maritime pine, *Pinus pinaster*; and *Eucalyptus* spp.) and shrubs (genus *Erica*, *Calluna*, *Ulex* and *Cytisus* (Carmo *et al.*, 2011; Scotto *et al.*, 2014).

1.4. Ecological consequences of fires

The ecological impact of fire on floral and faunal life-history traits varies along temporal and spatial scales as well as the interrelations between them. Moreover, wildfires result

from interactions of atmospheric conditions (e.g. temperature, humidity, etc.), ignition (natural or anthropogenic), vegetation (e.g. fuel moisture, species composition, etc.), and topography (e.g. elevation), thus, the impacts of fires may vary depending on the complexity of interactions between these factors.

Wildfires are recognized as key disturbance agents both at small (within-habitat) and large (ecosystems/landscapes) scales. Fires have both direct and indirect effects on individual organisms. Direct effects result from the fire itself causing emigration and/or mortality. Indirect effects result from shifts in the habitat structure and resource availability (e.g. food, shelter, etc.), and also modification of the landscape structure by creating a mosaics of fragmented habitats affected in different levels by fire (from unburnt to burnt habitats). Post-fire alterations of environmental conditions and habitat structure govern the short- and long-term temporal and spatial dynamics of fauna and flora (Izhaki, 2012).

Nevertheless, during their evolutionary development, flora and fauna have experienced changes in fire regimes. These different spatial and temporal patterns of disturbance are likely to have acted as profound selective forces resulting in organisms with life-history attributes that enable them to cope with fire regimes (e.g. in animals, Hutto, 2008; in plants, Pausas, 1997).

1.4.1. Impacts on fauna

Fire regimes and vegetation structure and composition form a direct feedback loop, where fire regimes shape patterns in the vegetation, and vegetation, in turn, affects fire regime attributes (e.g. frequency, severity). Animals are also involved within this feedback loop: animals affect vegetation structure and composition, and vegetation influence animal occupancy, survival, and reproductive rates by altering habitat characteristics. Therefore, wildfire has influenced the composition, structure and landscape patterns of animal's habitat (Sarà *et al.*, 2006; Haslem *et al.*, 2011).

Whelan *et al.* (2002) identified three levels on which fire affects animals: i) mortality and immigration/emigration; ii) post-fire viability; and iii) adaptation to fire.

First (i), fire can cause injury or death to individuals, or it can force individuals to move from (emigration) or into (immigration) the burnt area. Exposure to high temperature, toxic compounds of smoke, and oxygen depletion can cause mortality and injury. Despite the perception that wildfires are devastating to animals, fires generally kill and injure a relatively small proportion of animal populations (e.g. animals with low mobility, Couturier *et al.*, 2011). Fire affects animals mainly through habitat modification,

since vegetative structure spatially arranges the resources needed (food and shelter) (e.g. Santos and Poquet, 2010; Haslem *et al.*, 2011; Santos *et al.*, 2014). Generally, after the fire event, the burnt area is colonized by species associated with open areas, while species that depend mostly on more vegetated habitats avoid these areas. Post-fire responses of fauna are often interpreted using a framework of secondary succession (Friend, 1993; Fox *et al.*, 2003; Santos and Cheylan, 2013). Based on the 'habitat accommodation model' (Fox, 1982), species enter a post-fire succession when their habitat requirements are met, and leave or decrease in number when post-fire changes in vegetation or competitive interactions render it unsuitable (but see Nimmo *et al.*, 2014). Likewise, other factors are capable to influence faunal responses to fire, such as biotic interactions (e.g. predation; Torre and Díaz, 2004) or abiotic conditions (e.g. rainfall, Letnic *et al.*, 2004).

Second (ii), if enough individuals within a population survive, processes after the fire, such as starvation, predation, or immigration within the post-fire environment, will determine population viability (Russel *et al.*, 2003; Wilgers *et al.*, 2007). How humans burn or suppress fires determines which wildlife species can inhabit and reproduce in an area and therefore alter the community and species assemblages (Templeton *et al.*, 2001; Brotons *et al.*, 2005; Sarà *et al.*, 2006; Santos *et al.*, 2009; Valentine et al; 2012). Wildlife evolved with the fire regime of their habitat; therefore, if we change one, we affect the other. Nevertheless, there are a wide range of succession trajectories for animals but these will depend on species specific requirements and upon post-fire biotic and abiotic conditions in the burnt areas (Izhaki, 2012).

Lastly, the fire regime can modify a species over time to create adaptations to fire (i.e. evolutionary effects). Just like fire shaped plant traits to adapt to a certain fire regime (Keeley *et al.*, 2011), also animals have some cases of adaptation as in the case of the Black-backed woodpecker that is specialized in severely burnt forests (Hutton, 2008), or the rapid evolution of fire melanism in Pygmy grasshoppers (Forsman *et al.*, 2011).

The effect of fire on faunal diversity remains largely uncertain although there are some comprehensive studies upon vertebrates (e.g. Prodon *et al.*, 1987; Smith *et al.*, 2000; Sarà et al, 2006; Moreira and Russo, 2007; Fontaine and Kenedy, 2012; Izhaki, 2012; Pastro *et al.*, 2014). There are several explanations for such variation in the effects of fire: the inherently heterogeneity of fire (e.g. intensity and severity, Cleary *et al.*, 2004; Pastro *et al.*, 2011), and the habitat type or geographical location (e.g. Haslem *et al.*, 2012; Nimmo *et al.*, 2014; Pastro *et al.*, 2014). Also the choice of the study taxon can influence the findings of fire studies (Pastro *et al.*, 2011): for example, birds can evade from the fire front, but less likely will amphibians; endothermic and ectothermic

vertebrates may respond differently owing to their ecological requirements (Letnic *et al.*, 2004; Santos *et al.*, 2014).

1.5. Genetic consequences of fires

Environmental disturbance governs the dynamics and diversity of many of the world's ecosystems (Turner, 2010; see previous sections and references therein). However, despite the recognition that disturbance shapes biodiversity both at species and community levels (e.g. Petraitis *et al.*, 1989), its role as a driver of the patterns and distribution of genetic diversity is not well understood which makes this a major knowledge gap (Hughes *et al.*, 2008; Banks *et al.*, 2013). Here genetic diversity regards the metrics that quantify the magnitude of genetic variability at individual (e.g. observed heterozygosity), population (e.g. observed and expected heterozygosities, allelic richness), and among-population level (e.g. genetic differentiation measures such as F_{ST}, Analysis of Molecular Variance), and that are a fundamental source of biodiversity. Population genetics is a field of biology that studies the genetic variation within and across biological populations, and involves the examination of changes in the frequencies of genes and alleles in populations over space and time. Allele frequency in populations can change spatially and temporally under the influence of various evolutionary processes, particularly natural selection, genetic drift, mutation, and gene flow. Comparative analyses of spatial and temporal patterns in allele frequency provide an important entry point to identify the evolutionary forces that gave rise to them.

Genetic diversity provides the raw material for evolution by natural selection (Fisher, 1930) and adaptation to environmental changes (Pauls *et al.*, 2013). Genetic diversity has important consequences for all levels of biodiversity (Hughes *et al.*, 2008): in the fitness of individuals (e.g. Leimu *et al.*, 2006), the viability of populations (e.g. Templeton *et al.*, 2001), the adaptability of species to environmental change (e.g. Frankham *et al.*, 2002) and disease (e.g. Pearman and Garner, 2005), the structure of communities, and the function of ecosystems (Hooper *et al.*, 2005). Hughes *et al.* (2008) stated that as long as there is variation in ecologically important traits, the amount of genetic diversity at any level can have important ecological effects.

Banks *et al.* (2013) suggested three reasons why improving understanding of how disturbances influences the patterns of genetic diversity is important: i) disturbance patterns are changing rapidly in a human-modified world and will become an important driver of distribution of all levels of biodiversity; ii) the spatial and temporal patterns of genetic diversity can potentially tell us much about the demographic response of

populations to disturbance; and iii) disturbance history may be the major driver that shapes the patterns of genetic diversity in natural populations due to variations in demographic and biological processes that are known to influence the distribution of genetic diversity (e.g. bottlenecks, Brown *et al.*, 2013; social behaviour, Banks *et al.*, 2012). Similarly, Pauls *et al.* (2013) reviewed how global climate change affects genetic diversity and argue that in order to understand all consequences of climate change, one must study its consequences in all biodiversity levels, including the genes level. Because a large part of genetic diversity is selectively neutral, its distribution is shaped by mutation, migration, and genetic drift that arose from environmental and demographic changes imposed by disturbance (Holderegger *et al.*, 2006). Selective processes act on genetic diversity as a result of disturbances as well (by adaptation to new environmental conditions), but their genomic causes and consequences are often obscure in typical population genetic studies which study only neutral variation. Therefore, disturbances can influence genetic diversity either by selectively neutral demographic processes (e.g. directly due to mortality or indirectly by habitat suitability), or selective processes (Banks *et al.*, 2013).

Fire is a worldwide disturbance that has been shaping biomes (Bond *et al.*, 2005) and, therefore, flora and fauna (Izhaki, 2012), and which frequency is predicted to increase (Dury *et al.*, 2011). Although the ecological effects of fire have been studied in relative detail (see previous section and references therein), the effect of fire on genetic variation of natural populations remains largely underlooked, especially for animals (Steinitz *et al.*, 2012). Therefore, it is imperative to fill the knowledge gap. To understand the genetic consequences of fire, one must consider its ecological impact. In plants, fire affects in different ways the genetic diversity according to their mode of post-fire regeneration (resprouters or non-resprouters). Post-fire resprouting species have a high probability of surviving fires, thus the allelic frequencies and spatial genetic structure remains highly similar to the pre-fire population (Steinitz *et al.*, 2012). On the contrary, non-resprouters (i.e. seeders) die during fire events, but their seed bank is recruited shortly after fire which means that seedling will replace the lost genetic variation but the spatial clustering will be different from the pre-fire population (Segarra-Moragues and Ojeda, 2010). On animals, responses to fire are complex and species specific (Izhaki, 2012); therefore, the demographic effect of fire on animal populations depends on the fire characteristics (Pastro *et al.*, 2011) as well as species-specific dispersal and colonization abilities and ecological requirements (Whelan, 1995). One of the striking ecological effects of wildfires is mortality that will impose a bottleneck (reduction in population sample size) on the disturbed population. After the fire, another important biological process takes place: post-fire colonization. Population recovery stems from

survivors or by individuals from the outside (founding colonizers) (Robinson *et al.*, 2013). Both mortality and recruitment interact and thus, may influence the consequences of fire on within-population genetic diversity (Banks *et al.*, 2013). On one hand, the (genetic) bottleneck imposed by the fire (Whelan *et al.*, 2002; Banks *et al.*, 2011) produces a random loss of allelic diversity and in which the degree of loss will depend on the bottleneck degree and duration (Garza and Williamson, 2001) – severe bottlenecks tend to cause a major depletion of genetic diversity. Hence, the genetic composition of the disturbed population can vary greatly from this event on. On the other hand, the survival (which is a function from the disturbance degree and the ability to persist during the disturbance) or recolonizing rate (depending on the number of founder individuals and its dispersal ability) will further dictate the patterns of genetic diversity. The loss of allelic diversity could be lowered (or be inexistent) either if there is a high survival rate both on animals (e.g. endangered bird, Suarez *et al.*, 2012) or plants (e.g. resprouters, Steinitz *et al.*, 2012) or if there is a recruitment of individuals from multiple sources (e.g. post-fire specialist small mammal, Pereoglou *et al.*, 2013; lizard, Schrey *et al.*, 2011b). On the contrary, when the disturbed population remains small and isolated its persistence is affected likely due to non-random mating which leads to inbreeding depression and lower fitness. This is the case of the critically endangered tropical conifer *Araucaria nemorosa*, in which frequent fires lead to elevated levels of inbreeding in the juveniles (Kettle *et al.*, 2007).

After the recovering process takes place (either by survival or colonization), fire also directly affects genetic differentiation among populations (e.g. usually measured by the F_{ST}) through its influence on genetic drift and migration (Brown *et al.*, 2013). Genetic drift is responsible for the random alteration of allele frequencies, which in turn increases genetic differentiation between populations due to population turnover, especially if they are small and without gene flow (e.g. lizard, Templeton *et al.*, 2001; tree, De-Lucas *et al.*, 2009; bird, Brown *et al.*, 2013). Conversely, if migration is facilitated between populations, then the populations that are interchanging individuals will become more similar genetically due to increased gene flow (e.g. wind-mediated pollen dispersal in pinus, Shohami and Nathan, 2014; lizards, Schrey *et al.*, 2011a; fire-specialist bird, Pierson *et al.*, 2013).

Indirectly, fire can also affect genetic diversity of animals through its influence on the spatial and temporal patterns of habitat suitability (e.g. resource availability) and community composition and movement of animals (Sarà *et al.*, 2006; Haslem *et al.*, 2011; Banks *et al.*, 2013). For example, it is known the case of the collared lizard (*Crotaphytus collaris*) in the Missouri Ozarks in which fire suppression reduced their habitat and disrupted gene flow between metapopulations (Templeton *et al.*, 2001). Due

to isolation and drift, this species even faced extinction by 1980; until the beginning of prescribed fires when the populations became again a stable metapopulation (Templeton *et al.*, 2011). Similarly, recently burnt sites were preferred by open-habitat or early stage species which, in turn, increased its local density and genetic variation (e.g. lizard, Schrey *et al.*, 2011b; small mammal, Pereoglou *et al.* 2013). The contrary is also true, a decrease in the genetic diversity due to habitat fragmentation patterns imposed by fire both in animals (e.g. butterfly, Fauvelot *et al.*, 2006) and plants (e.g. fire-dependent shrub, England *et al.*, 2002).

Apart from selectively neutral demographic processes, Banks *et al.*, (2013) also suggests that natural selection can influence genetic diversity in disturbance scenarios when disturbances are frequent. There can be a local adaptation to particular regimes, as such, in fire-prone environments plants showed to have adaptive traits ('fire-resistance traits') owing to their evolution under frequent fires (Keeley *et al.*, 2012). For instance, topographically mediated variation in fire regimes can influence spatial patterns of selection for fire response traits in plants, such as resprouting or obligate seeding (Clarke, 2002). In animals there are also some cases of adaptation as in the case of the Black-backed woodpecker that is specialized in severely burnt forests (Hutton, 2008), or the rapid evolution of fire melanism in Pygmy grasshoppers (Forsman *et al.*, 2011).

The incorporation of knowledge of both direct, in particular the demographic processes (Steinitz *et al.*, 2012), and indirect effects of fire is important to understand the underlying processes shaping genetic diversity produced by disturbances and perhaps attempt to predict these effects (Banks *et al.*, 2013). Furthermore, Steinitz *et al.* (2012) highlights the fact that including control populations not affected by fire is essential for differentiating between fire and other temporal effects. They further advice that one must consider the ecology of species and which are the ecological effects of fire on that species (Steinitz *et al.*, 2012).

1.6. Environmental stress and developmental stability

Developmental stability is defined as the ability of an organism to buffer its genetically predetermined phenotype development against genetic or environmental disturbances encountered (Waddington, 1942; Markow, 1995). When organisms fail to buffer such disturbances, it may display signs of development instability. The origin of the disturbance is assumed to be genetic, environmental, or the product of a genotype-environment interaction (Markow, 1995). Developmental instability is often assessed by the level of fluctuating asymmetry (FA), which corresponds to the small and random

differences between the right and left sides of an otherwise bilaterally symmetrical character (Van Valen, 1962; Palmer and Strobeck, 1986; Zakharov, 1990). The underlying assumption of FA is that the development of the two sides of a bilaterally symmetrical organism is influenced by identical genes and, therefore, non-directional differences between the sides must be environmental in origin and reflect accidents occurring during development (Waddington, 1942). Because developmental stability acts to suppress such accidents, fluctuating asymmetry will reflect the efficiency of developmental stability mechanisms but also the intensity of disturbance factors (Van Valen, 1962; Palmer and Strobeck, 1986) – the larger the FA, the lower the development stability. Detailed descriptions of analyses as well as constraints can be found in Palmer and Strobeck (1986) and Palmer and Strobeck (1992) and will not be discussed here.

Interest in FA originated due to of its potential for measuring population-level stress. At the population level, if one population is more asymmetrical (less stable) than another population for one character, there is a tendency that it will also be more asymmetrical for other characters (Soulé, 1967). Such differences in stability between populations have led to the widespread use of developmental stability analysis as a technique for identifying and characterizing populations subject to systematic stress (Leary and Allendorf, 1989; Parsons, 1992). Nevertheless, FA can be also an indicator of individual quality, mainly studied regarding the fitness (reviewed in Møller, 1997) and on sexual selection (reviewed in Møller and Pomiankowski, 1993). Basically, FA measures the adaptive value or quality of an individual. Two types of stress can produce a decrease in the quality of the individual or population – genetic (e.g. inbreeding, reduced heterozygosity, etc.), environmental change (e.g. temperature, pollution, etc.), or the interaction of both (Palmer and Strobeck, 1986; Markow, 1995 and references therein). Therefore, FA can serve as a 'biological monitor of environmental and genomic stress' (Parsons, 1992). One of the mechanisms explaining the maintenance of development stability have a genome-wide basis (Clarke, 1998): the heterozygosity theory, in which the genetic variation is argued to have a buffering role by increasing biochemical diversity and thus enabling a dynamic stable developmental pathway in changing environments. Likewise, developmental instability can be enhanced by a decreased ability to cope with environmental changes due to low genetic diversity (e.g. inbreeding and genetic drift). Periods of rapid environmental change can alter individual energy expenditure during development and thereby increase phenotypic variation in populations (Hoffmann and Parsons, 1997). Such increased variation may be evident in deviations from optimal development in morphological traits, which is expressed as increased asymmetry (Palmer and Strobeck, 1986). Under stressful conditions, the development of symmetrical traits might be more difficult to achieve and Van Dongen

and Lens (2000) pointed out the importance of knowledge about those conditions where developmental stability reliably reflects stress.

The most direct effect of wildfires is the consumption of vegetation. The intensity and severity of the fire will determine the degree of burnt conditions. By reducing the overstory and understory plant density, wildfire create a new state of the environmental conditions on the burnt area that are different from the pre-fire conditions. The post-fire openness of the habitat allows for increased insolation to reach the ground surface, which leads to an increase of soil and above-ground air temperature. In turn, during the night there is a more rapid heat loss and cooler soil and air temperatures. Moreover, with a reduction in cover (i.e. shadow) and increasing temperatures, the humidity and moisture will also be affected negatively. Therefore, in burnt areas it is expected an environmental stress due to increasing temperatures and decreasing humidity (i.e. the area becomes more arid), but also due to higher amplitude of temperatures. This environmental stress can affect animals inhabiting burnt areas in two different ways: surviving animals do not cope with the increasing harsh conditions and lack of resources and emigrate, or die (discussed in previous section); while animals that are under embriogenic development during post-fire conditions may be morphologically affected, or not. The morphological characteristics of the organisms are essential to the survival and successful reproduction of individuals (i.e. fitness). Thus, some morphological traits have important known biological functions such as reproductive behaviour (e.g. femoral pores in lizards; Lopéz *et al.*, 2002), or habitat use that will allow the viability of the individuals (e.g. subdigital lamellae; Glossip and Losos, 1997). Although it is not clear whether asymmetry in such traits is traduced in fitness disadvantages *per se* (e.g. asymmetry in femoral pores not disturbing reproduction). Assessing the FA of populations in burnt areas through the use of morphological traits is a useful approach to evaluate the effect of the post-fire environmental conditions on the individual quality and stability of the population. As stated earlier, the genetic diversity can also affect the degree of the stable development of organisms, hence taking this factor into account will also be important. This will be possible by testing the differences of the degree of FA and levels of genetic diversity between populations of burnt habitats and populations of unburnt habitats (i.e. control).

1.7. *Podarcis hispanica* complex

Wall lizards of the genus *Podarcis* (Squamata: Lacertidae) are among the most conspicuous, abundant and widely distributed reptiles in Europe and North Africa. Thus,

it is reasonable that it is the most commonly studied lizard genus regarding ecological and evolutionary studies from individual to community level at multiple spatial and temporal scales (Camargo *et al.*, 2010). Several phylogenetic studies exist regarding the entire genus (e.g. Harris and Arnold, 1999; Harris *et al.*, 2005), on a specific geographical group (e.g. Iberian and North African wall lizards, Pinho *et al.*, 2007; Balkans wall lizards, Poulakakis *et al.*, 2005), or at the species level (e.g. *P. lilfordi*, Terrasa *et al.*, 2009; *P. muralis*, Michaelides *et al.*, 2015). The genus currently comprises 23 fully recognized species and exhibits a circum-mediterranean distribution (Arnold, 1987; Uetz *et al.*, 2014, http://www.reptile-database.org).

The systematics of the genus is unstable and is still being discussed (e.g. some forms of *P. hispanica* are now called *P. guadarramae*; Geniez *et al.*, 2014). In particular, the *Podarcis* wall lizards from the Iberian Peninsula and North Africa (except for *P. muralis*), that represent a characteristic case of cryptic species complex (Sá-Sousa, 2000; Harris and Sá-Sousa, 2002), have a long history of taxonomic revisions and instability at the specific and subspecific level (Pérez-Mellado, 1998; Carretero, 2008). Traditionally, only two endemic species were recognized in this complex – *P. bocagei* (Seone, 1884) in westernmost Iberian Peninsula, and *P. hispanica* (Steindachner, 1870) throughout the Iberian Peninsula and North Africa (Arnold *et al.*, 1978). However, although being morphologically similar, these species exhibited substantial levels of intraspecific variation (Arnold *et al.*, 1978). Since then, extensive research using molecular techniques both for phylogenetic inference and phylogeographic analyses with mtDNA and nuclear markers (e.g. Harris and Sá-Sousa, 2002; Harris *et al.*, 2002; Pinho *et al.*, 2006; Pinho *et al.*, 2007) coupled with morphological differences (e.g. Sá-Sousa *et al.*, 2002; Kaliontzopoulou *et al.*, 2012a) have contributed to disentangle the taxonomy of this complex. Normally, the lineages observed in mtDNA are also recovered by nuclear markers (Pinho *et al.* 2007; but see Renoutl *et al.*, 2010) and morphology (Kaliontzopoulou *et al.*, 2012a).

Currently, seven species are recognized within the *P. hispanica* complex (Geniez *et al.*, 2007; Renoult *et al.*, 2010; Geniez *et al.*, 2014): *P. bocagei* (Seoane, 1984), *P. carbonelli* (Pérez-Mellado, 1980), *P. vaucheri* (Boulenger, 1905), *P. liolepis* (Boulenger, 1905) (former *P. hispanica* morphotype 3 in Pinho *et al.*, 2006), *P. guadarramae* (Boscá, 1916) (former *P. hispanica* morphotype 1 in Pinho *et al.*, 2006) and. *P. virescens*, (former *P. hispanica* morphotype 2 in Pinho *et al.*, 2006). Finally, *P. hispanica* includes all remaining forms described within the complex and constitutes a polyphyletic taxa. In terms of distribution, Kaliontzopoulou *et al.* (2011) provides a comprehensive summary of the distribution of the various forms of the *P. hispanica* complex (although lacking more recent updates, therefore see Renoult *et al.*, 2010 and Geniez *et al.*, 2014). The

majority of lineages are parapatric, with some exceptions in the pairs *P. bocagei* - *P. guadarramae guadarramae* and *P. carbonelli* - *P. virescens*, which are sympatric or even syntopic. Sympatry is rare but also present between *P. carbonelli* and *P. guadarramae* in the Iberian Central System and between *P. vaucheri* and *P. carbonelli* in Doñana National Park (Carretero, 2008).

In terms of morphology, the species of this genus are highly variable in size, shape, scalation and colour patterns (Arnold *et al.*, 1978; Harris and Sá- Sousa, 2001; Sá-Sousa *et al.*, 2002; Kaliontzopoulou *et al.*, 2012a). Moreover, this variability is not only present between species but also between populations (Kaliontzopoulou *et al.*, 2012b). Sexual dimorphism is strong in the genus (Kaliontzopoulou *et al.*, 2007, 2008).

1.7.1. Case study: *Podarcis guadarramae*

Podarcis hispanica type 1 (type 1A + type 1B) was elevated to species level by Geniez *et al.* (2014), being now named *Podarcis guadarramae.* The type 1A and 1B remained conspecifics despite the suggestions pointing to distinct species (Geniez *et al.*, 2014). Type 1A is now subspecies *P. guadarramae lusitanica* (Geniez *et al*., 2014), while type 1B is subspecies *P. guadarramae guadarramae* (Boscá, 1916). The former occurs in northern Portugal and north-western Spain while the latter is only found in the Central Iberian Mountains in Spain (Pinho *et al.*, 2006). What prevented the elevation to specific level of both subtypes was the lack of external morphological identification features leading to a lack of information on contact zones (Geniez *et al.*, 2014). Full description and comparisons can be assessed in Geniez *et al.* (2014).

In Portugal, *Podarcis guadarramae lusitanica* is only found in north-western part where it is largely sympatric with *P. bocagei* in the north-west of its range (Loureiro *et al.*, 2010) although reproductive isolation is nearly complete (Arntzen and Sá-Sousa, 2007). *Podarcis guadarramae* is also sympatric, and sometimes syntopic, with *P. carbonelli* in the north of Portugal (Loureiro *et al.*, 2010) and globally parapatric with *P. virescens* in Portugal (Sá-Sousa, 2000), however with some syntopic observations (Pérez-Mellado, 2010). The coexistence of species are usually mediated by ecological segregation at habitat (*P. guadarramae – P. bocagei*; Sá-Sousa, 2000), trophic or time (*P. guadarramae – P. carbonelli*; Pérez-Mellado, 1983) level.

The Iberian wall lizard, *P. guadarramae*, is an insectivorous and small (50–70 mm adult snout-to-vent length) diurnal lacertid lizard. While most *Podarcis* species use either rocks, trunks, vegetation or bare ground for thermoregulation, foraging and shelter (Carretero, 2008), *P. guadarramae* shows a degree of habitat specialization since it is

very saxicolous (as *P. hispanica* type 1 in Sá-Sousa, 2000). Its depressed body shape and flattened skull facilitate entrance into narrow, irregular crevices. It is common at rocky habitats or artificial walls (Martín-Vallejo *et al.*, 1995; Diego-Rasilla and Pérez-Mellado, 2003) where individuals aggregate around favourable areas with rock crevices and less vegetation (Gil *et al.*, 1988; Lopéz and Martín, 2002; Diego-Rasilla and Pérez-Mellado, 2003). There is only small territoriality between males, where bigger males dominate smaller males (Carretero *et al*., 2015). Behavioural thermoregulation in *P. guadarramae* is mainly characterized by the adoption of basking postures and shuttling between sun and shade (Díaz *et al.*, 1996). More information regarding this species can be assessed in Carretero *et al*. (2015).

Figure 1.2. Specimen of *Podarcis guadarramae lusitanica*.

1.8. Objective and work hypotheses

This study aims to understand how a repeated fire regime characteristic of Northern Portugal affects populations of *Podarcis guadarramae* at two different, yet interacting, levels: genetic and morphological. Therefore, we used two types of data to address both issues. Both approaches are examined using five replicate pairs of populations in which each pair had one population from an unburnt area (hereafter named UN population; control) and one population from a burnt area (hereafter named BU population). The design accounted for burnt populations with different levels of disturbances (i.e. number of fires, time since the last fire) in order to have a disturbance gradient.

Besides *a priori* knowledge of occurrence of fire (BU) or not (UN), we measured the environmental conditions that most affect reptiles (temperature and humidity, Angilletta,

2009; Osojnik *et al.*, 2013) in order to test the assumption that our sampling areas had different environmental conditions depending on its condition (UN or BU), which was a prerequisite for further analysis (see below). We asked the following question:

i) Are UN areas environmentally different from BU areas?

Fires reduce the canopy, therefore, the remaining features of the habitat such as rocks, logs, or bare ground are more exposed to sun exposition. We expect that this increased solar radiation in BU areas produce higher temperature of the habitats which, in turn, cause a decreasing in relative humidity. Therefore, BU areas will have higher temperature and lower humidity, with higher amplitude ranges, while UN will feature lower temperature and higher humidity, both with narrower ranges.

Genetic diversity is the raw material that provides the ability to cope and adapt to changing ecological disturbances. Disturbances, such as fire, shape the distribution of species, thus their genetic diversity (Banks *et al.*, 2013). Direct mortality and post-fire recruitment may modulate the consequences of fire on within-population genetic diversity (Banks *et al.*, 2013). Based on these premises we want to answer the following questions:

i) How does the repeated wildfire regime affect the genetic diversity of populations?

We expect that a repeated fire regime produces an impact on the genetic diversity of *P. guadarramae* by decreasing the population size and, therefore reducing the number of alleles and heterozygosity. We further suspect that it will vary according to the degree of disturbance that populations experienced, with populations that experienced higher number of fires having lower genetic diversity due to recurrent bottlenecks and with populations recovering from more recent fires showing the same trend because of low population sizes and recent recolonization processes (i.e. founder effects). For these reasons, we also predict that populations exposed to fire will exhibit genetic signatures consistent with recent bottlenecks and recent immigration, whereas these signatures will be generally absent from stable (i.e. unburnt) populations.

ii) Can we genetically distinguish populations between UN and BU populations?

We predict that fires are capable to produce a stochastic shift in the genetic diversity of populations affected by reducing the population size (thus, gene diversity)

and by genetic drift. Therefore, we expect that this probable change in the genetic diversity in each fire event will further enhance the differentiation of BU populations from UN populations. The UN populations are supposedly free from this process (bottleneck), therefore will remain more similar, although with some degree of differentiation due to genetic drift of each population.

iii) Does fire affect the genetic structure of *Podarcis* populations?

We hypothesize that fire could have the capability to influence the genetic structure of populations due to the impact that fire has over the genetic diversity and differentiation of populations, even at the population pairwise level (UN-BU) – fine scale structure. We expect that fire disrupts the spatial structure of populations by altering the relationship of genetic with geographic distances due to demographic processes that arose from fire and pos-fire population dynamics.

Post-fire environmental conditions become harsher since there is increased solar radiation that leads to a higher soil and above ground air temperature and lower humidity. Both temperature and humidity have been demonstrated to be important to the correct egg development and consequent phenotype in hatchling reptiles (e.g. Shine *et al.*, 1997; Shine, 2004; Braña and Ji, 2007; Monasterio *et al.*, 2013; but see Ji and Braña, 1999). However, these studies do not account for the developmental stability by measuring deviations to bilateral symmetry but rather measuring body size, shape and locomotor performance. Nevertheless, reptiles have been more or less used on developmental instability studies (see review in Laia *et al.*, 2015 and references therein). Here, we want to understand if post-fire conditions (higher temperature, lower humidity) affect the individual quality and population stability by examining fluctuating asymmetry (FA) of three morphological traits (with and without biological functions). Also, developmental stability has a genetic basis in which genetic diversity can buffer environmental stress by allowing individuals to, supposedly, better adapt to changes. Particularly, the Heterozygosity Theory says that heterozygosity (both at individual and population levels) is negatively correlated with asymmetry (Clarke, 1998). Specifically, we want to answer these questions:

i) In which populations, UN or BU, the degree of asymmetry (thus, developmental instability) is higher?

We hypothesise that the degree of developmental instability, measured by the FA, is higher in the BU populations due to stressful post-fire environmental conditions.

ii) Is FA trait-specific, and is it related to their functional importance?

We expect that developmental stability is trait specific, this is, traits that have biological functionality (adaptive and highly conserved) would show lower degree of FA than functional ones.

iii) Is FA, at both individual and population level, correlated with genetic diversity?

Following the Heterozygosity Theory, we expect that individuals that have higher genetic diversity (i.e. heterozygosity) have lower degree of FA. Similarly, populations with higher genetic diversity are expected to have lower degree of FA and therefore, be more stable and viable under environmental stress.

In summary, we expect that repeated fires have a detrimental effect on *Podarcis guadarramae* populations, both at the genetic and morphological levels.

To our knowledge, this study is a novelty. The impact of wildfires on genetic diversity was never assessed in lizard populations in the Mediterranean Basin (see Schrey *et al.*, 2011a in the United States, and Ujvari *et al.*, 2008 and Smith *et al.*, 2014 in Australia). Also, no study has specifically tested the effects of post-fire environmental conditions on the developmental stability of fauna (for flora see Graham *et al.*, 2012, although they only account for fire indirectly). Therefore, the results are valuable information to help filling the gap of genetic and ecological impacts of fires on natural populations.

2. Materials and Methods

Content

2.1. Study area

This study was performed with several populations of *Podarcis guadarramae* in northern Portugal, in western Mediterranean basin. The study area was confined by the latitudes 41º05' to 42º15' N and longitudes -8º90' to -7º76' W (**Fig. 3**). Since there are several locations sampled, moderate variability can be observed regarding the climatic and environmental characteristics and systematic description of the study area according to the different locations will not be done. Instead, general habitat description will be given.

Northern Portugal is located in the transition between the Mediterranean and Eurosiberian biogeographic zones close to the Atlantic coast (Metzger *et al.*, 2005). Essentially, the study area has a transitional climate between Atlantic (NW) to Mediterranean (NE). Average annual precipitation ranges from approx. 1200 mm at the littoral to 2800 mm at the upper elevations in the interior, mostly falling between October and April and with 0–2 rainless months. The average annual temperature is 14.5 ºC, mean maximum and minimum temperatures are respectively 25 ºC in July and 4 ºC in January (Ribeiro *et al.*, 1987).

Forests are very fragmented but the characteristic species are *Quercus robur*, *Q. pyrenaica*, *Acer pseudoplatanus*, *Pyrus cordata* and *Ilex aquifolium* (Costa *et al.*, 1998). The most common type of vegetation is scrub communities composed by genus *Erica*, *Calluna*, *Ulex* and *Cytisus*.

2.2. Experimental design

To test how the repeated fire regime that characterizes the study area affect both genetic and morphologically the wall lizard *Podarcis guadarramae*, a paired-population sampling scheme was chosen. Each location sampled was composed by two populations with contrasting conditions. Here, the condition refers to the disturbance imposed by fire – burnt population (BU) - or the lack of it – unburnt population (UN; control population). Within the study area defined (**Fig. 3**), five locations (equivalent to 10 paired-populations), were selected based on a digital national cartography of burnt areas in a shapefile with areas burnt from 1975 to 2009 (ICNF by JMC Pereira, 2014) and from 2009 to 2013 (http://www.icnf.pt/portal/florestas/dfci/inc/info-geo). Therefore, this study had five replicates in which each location is an independent replicate. The selection of each location involved the following criteria:

(i) proximity of UN and BU in order to minimize spatial structuring between populations;

(ii) abundance of *P. guadarramae*;

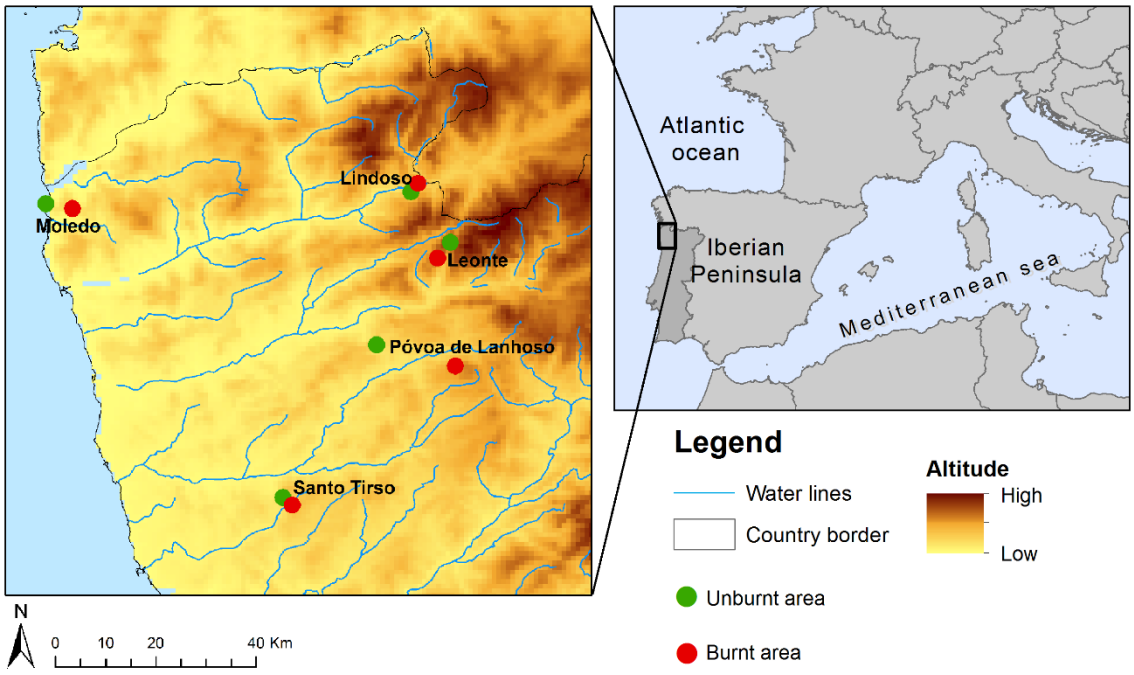
(iii) number of fires;

(iv) burnt areas larger than 20 hectares.

Site selection was constrained by the presence of *P. guadarramae*, particularly on unburnt areas which are becoming scarce in northern Portugal. Some unburnt populations were sampled inevitable on areas that have burnt. Nevertheless, we attempted to do it on areas that had the smallest number of fires as possible, as well as with low intensity fires and that occurred long time ago. For these logistic reasons, some UN-BU pairs are separated by a few kilometres (see description of locations below).

In reality, the populations sampled form a gradient from less burnt to extensively burnt areas (different number of fires and time since the last fire). Therefore, to take advantage of such effect some analyses were done regarding this gradient in which the initial paired structure was dissociated to be able infer general patterns.

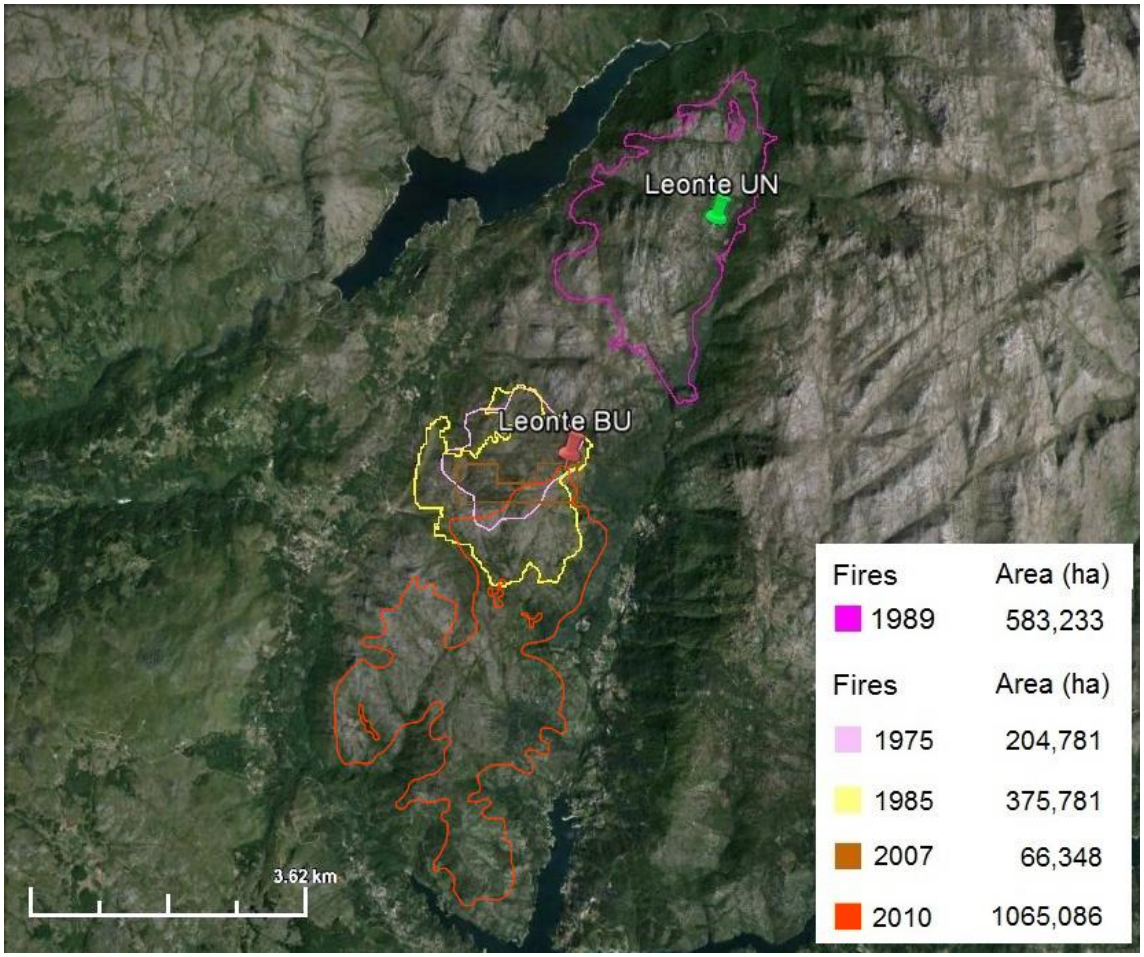
Figure 2.1. The study area is located in the NW extreme of Portugal which is the most affected area by fires in Europe. Each location sampled is formed by two populations with opposite conditions, one unburnt (UN, green) and one burnt (BU, red).



2.2.1. Leonte

The first sampled location was Leonte in the Peneda-Gerês National Park (PNPG) in which populations are apart by 3.64 km (**Fig. 4**). The unburnt population was sampled on Mata da Albergaria, a fully protected site within the park due to its natural biodiversity. Despite its pristine appearance, this site was burnt once in 1989 (**Fig. 4**), at least since there are records. Leonte BU have one of the smallest fire-history (*n* = 4; **Fig. 4**) of the burnt populations sampled, which is not surprising given its proximity to villages which result in higher level of detectability and rapid action from fire-fighters. Also, the area is managed with prescribed fire (low intensity) which aims to reduce fuel loads and avoid wildfires in summer.

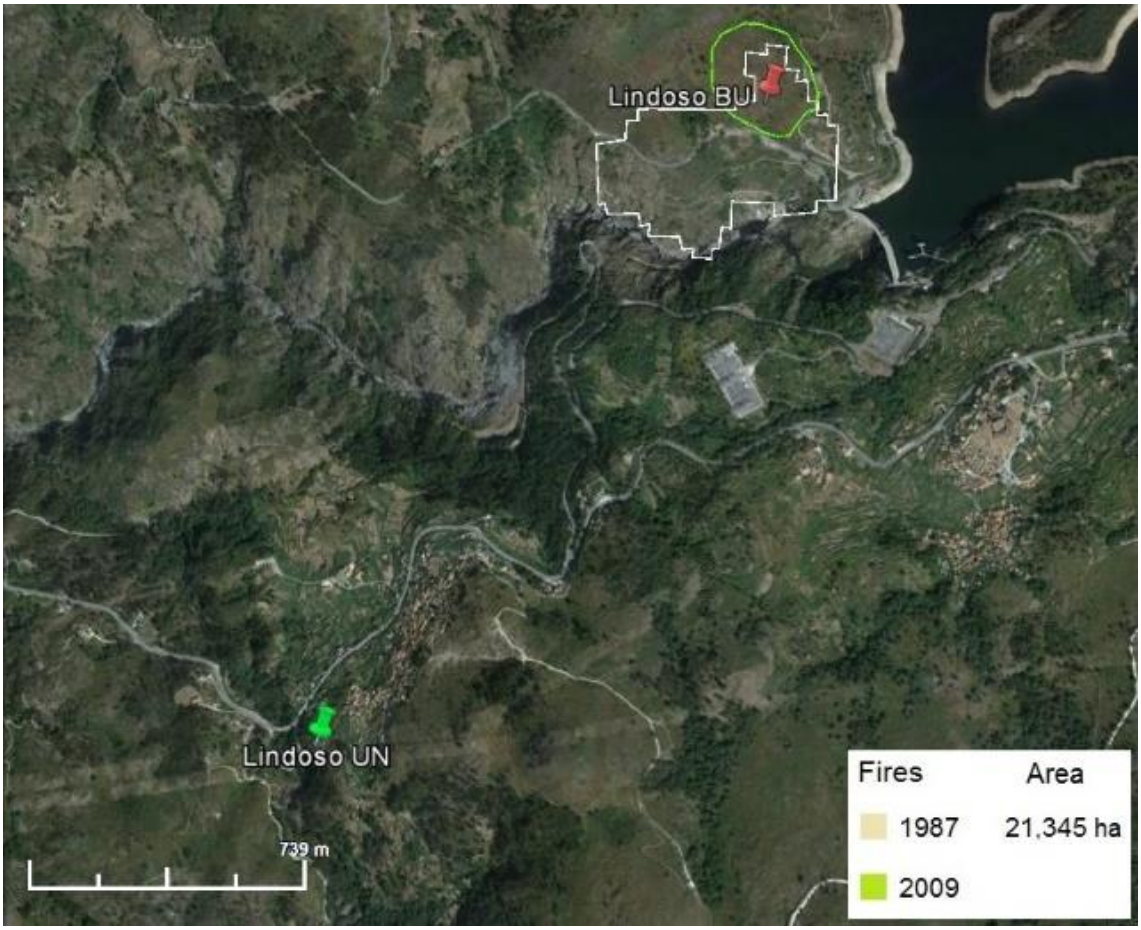
Figure 2.2. Fire-history of Leonte populations sampled (UN and BU). The pinpoints correspond to the centre of the sampling areas. Colored lines represent the different fires that affected these sites with respective area burnt in hectares.



2.2.2. Lindoso

Populations at Lindoso are also located within the PNPG and are 2.01 km apart and separated by the Lima River (**Fig. 5**). The unburnt population was sampled in the rural area of Parada in which the main habitat of *P. guadarramae* was the rock walls that separates properties. The burnt population was sampled just near the Alto Lindoso dam. This BU population have the lowest fire-history of the BU populations sampled (n = 2). The national fire database only reported one fire in the BU area in 1987 (**Fig. 5**) although polygons of more recent fires are located near the sampling point. Our fieldwork analysis evidenced a more recent wildfire. From Google Earth it was detected by eye that between 2006 and 2010 the BU area was more open with more evident rocky areas visible which suggests that there was at least one fire during this period. Therefore it was additionally considered another fire in 2009 (**Fig. 5**). The number of fires considered could be however underestimated. The more likely explanation is that that particular area was burnt to re-growth of vegetation to livestock grazing by prescribed fires. The main habitat was mainly characterized by rock outcrops interspersed in scrubland; this landscape structure is evidence of an intense fire-history.

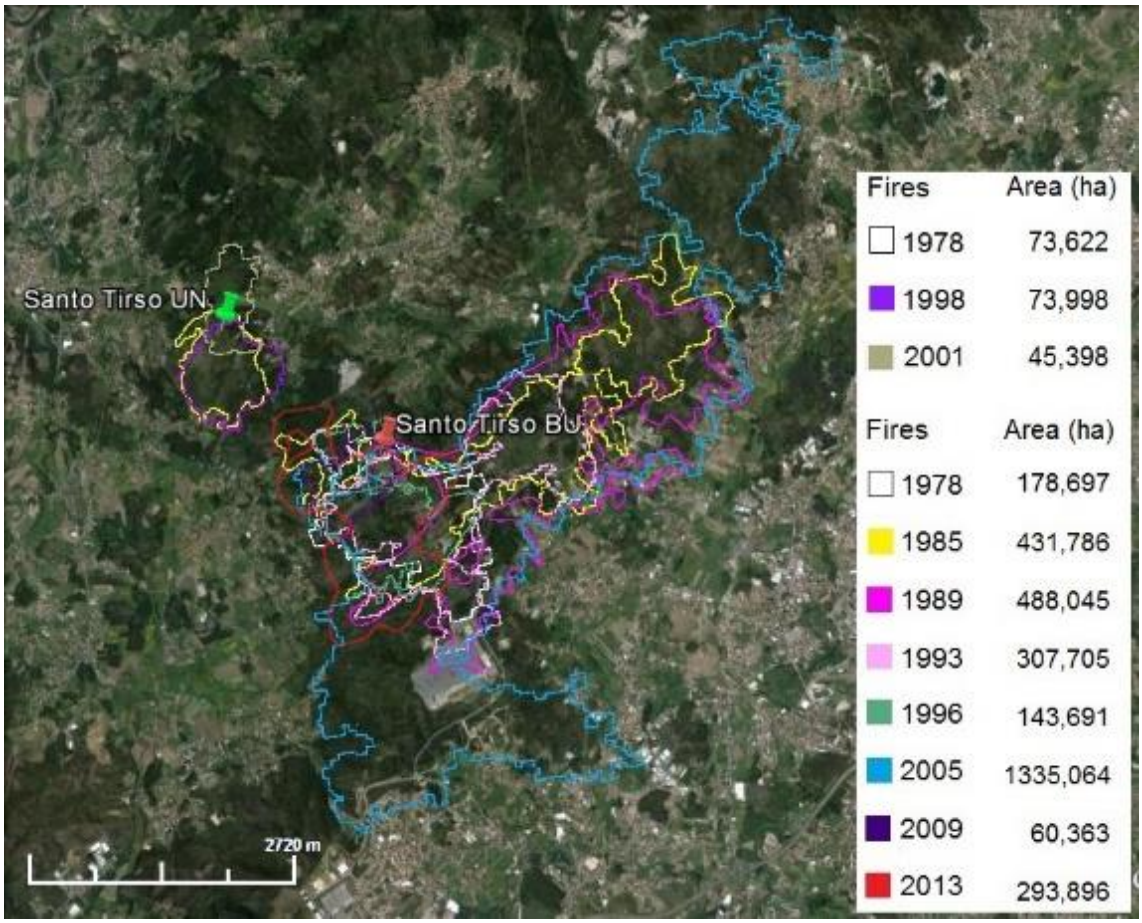
Figure 2.3. Fire-history of Lindoso populations sampled (UN and BU). The pinpoints correspond to the centre of the sampling areas. Colored lines represent the different fires that affected these sites with respective area burnt in hectares.



2.2.3. Santo Tirso

Santo Tirso is the southernmost location sampled in this study (**Fig. 6**). The UN and BU populations are distant by 2.08 km. The UN population is situated in ruins of an ancient roman city (Castro de Monte Córdova), therefore the habitat is more open and composed by walls and small scrubs. Just like Leonte, the unburnt population was affected by fires. This site with the presence of *P. guadarramae* was the most proximate from the BU site that had the lowest fire-history. Nonetheless, the fires were of only low severity (small size) with the last fire in 2001 (**Fig. 6**). Moreover, *Quercus suber* were examined to detect fire scars on the cork and only scars were found in the down parts of the trunk, suggesting low intensity surface fires. Given these evidences of very low severity fires we suspect that perhaps these had only marginally affected the UN population. In contrast, the BU population has a fire-history with multiple fires (*n* = 8; **Fig. 6**). The area was predominantly composed by large rocky areas and some low vegetation as a consequence of frequent fires and the fact that the last fire was the year before the sampling.

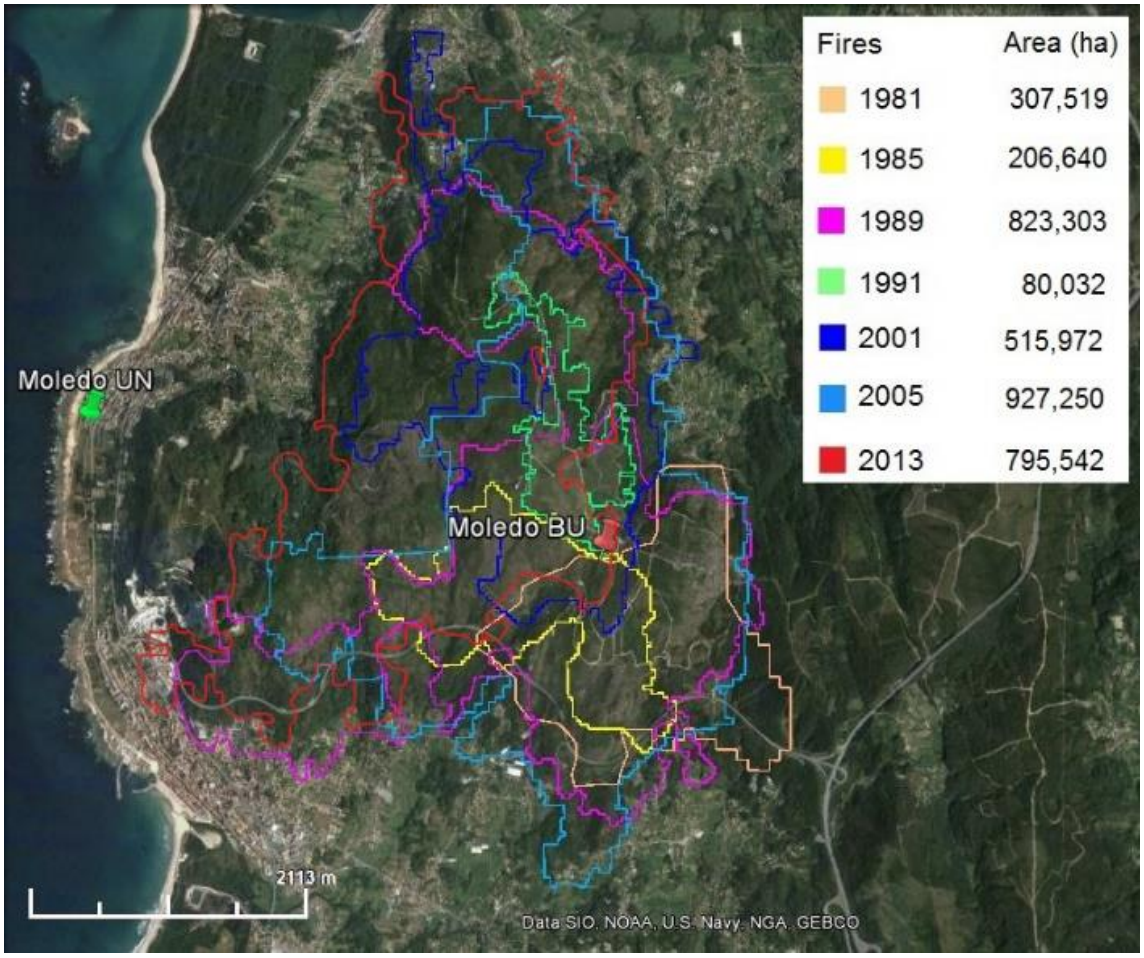
Figure 2.4. Fire-history of Santo Tirso populations sampled (UN and BU). The pinpoints correspond to the centre of the sampling areas. Colored lines represent the different fires that affected these sites with respective area burnt in



2.2.4. Moledo

Moledo is located in the westernmost extreme of the study area (**Fig. 7**), which in turn has a different climate from the other locations attributed to its littoral position with mild temperatures during all year. The UN-BU pair of populations was 4.07 km apart. The UN population was sampled in a moderately urbanized site at Moledo Beach in which individuals were found in isolated rocks and rock walls that separate small plantations fields. The BU population has a high fire-history (*n* = 7; **Fig. 7**) and in which the last fire was on the year before sampling (2013; **Fig. 7**). Thus, the habitat is still recovering from that fire and is mainly composed by rocks and shrubs. This pair shows the most different habitat and climate.

Figure 2.5. Fire-history of Moledo populations sampled (UN and BU). The pinpoints correspond to the centre of the sampling areas. Colored lines represent the different fires that affected these sites with respective area burnt in hectares.



2.2.5. Póvoa de Lanhoso

The last location sampled was Póvoa de Lanhoso in which UN population and BU population were separated by 12.47 km (**Fig. 8**). The reason for such geographic distance is that we did not find closer UN sites with *P. guadarramae* lizards. The UN population was sampled in the surroundings of the Póvoa de Lanhoso Castle which corresponds to an area with rock walls, isolated big rocks, as well as small scrubs. The BU population, along with Santo Tirso BU, had the highest fire-history across the sampled populations (*n* = 8; **Fig. 8**). The BU population was sampled at a mountain top in which the dominant vegetation form was low scrubland with rocky outcrops in-between.

Figure 2.6. Fire-history of Póvoa de Lanhoso populations sampled (UN and BU). The pinpoints correspond to the centre of the sampling areas. Colored lines represent the different fires that affected these sites with respective area burnt in hectares.

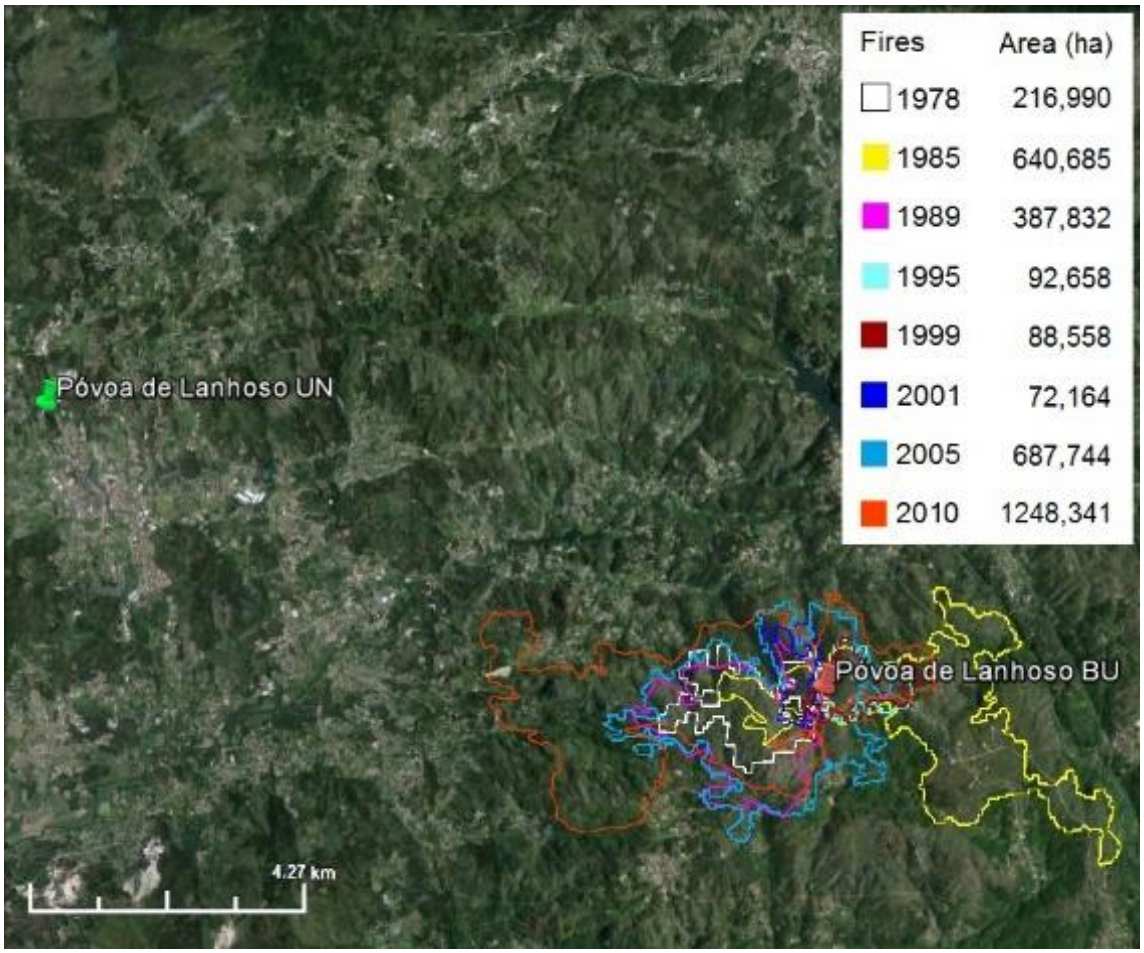


Table 2.1. Summary of fire-history of each population sampled. The time since the last fire (TSLF, in years) regards the Portuguese records (from 1975 to 2013). Therefore, the sites that have never been burnt since 1975 have a time since the last fire of 39 years.

Population	Number of fires	TSLF
Leonte UN	1	25
Leonte BU	4	4
Lindoso UN	0	39
Lindoso BU	2	4
Santo Tirso UN	3	12
Santo Tirso BU	8	1
Moledo UN	0	39
Moledo BU	7	1
Póvoa de Lanhoso UN	0	39
Póvoa de Lanhoso BU	8	4

2.3. Sampling methods

Fieldwork to collect samples was done between July-October of 2014. Four unburnt locations have *P. guadarramae* and *P. bocagei* occurring in sympatry (Leonte, Lindoso, Santo Tirso and Moledo); morphological diagnostic characters such as size, colour pattern and head shape were carefully checked to avoid species misidentification. Each population was sampled for approximately 20 individuals (**Table 2**) since this number was considered sufficient to perform population genetics analyses (Hale *et al.*, 2012). A total of 201 individual samples were collected for this study.

Individuals were captured within the area previously defined using a noose or by hand. Sampling syntopic individuals was avoided as well as sampling juveniles. Since this study is being conducted at the population-level, the geographic coordinates of each individual were not collected. The next steps were only performed when all the individuals for the population in question were sampled in order to avoid recaptures. Several high-resolution photographs of the individuals were taken regarding the traits that would be analysed between populations (dorsal head, cloacal area, and fourth toe of the hindlimb on both sides; for further information see below in section 2.6.). The tail tip of each individual was collected and stored in 96% ethanol at room temperature.

Table 2.2. Number of individuals sampled in each population.

Population	Sample size
Leonte UN	21
Leonte BU	20
Lindoso UN	20
Lindoso BU	17
Santo Tirso UN	20
Santo Tirso BU	21
Moledo UN	20
Moledo BU	22
Póvoa de Lanhoso UN	20
Póvoa de Lanhoso BU	20
Total	201

2.4. Environmental characterization of sampling areas

One of the premises of this study is that populations with contrasting conditions regarding the occurrence of wildfires would, in turn, be submitted to different environmental conditions. Wildfires produce a reduction in canopy, therefore, the remaining features of the habitat such as rocks, logs, or bare ground are more exposed to sun exposition. This increased solar radiation, supposedly, increase the temperature of the environment which in turn causes a decreasing in relative humidity. Therefore, a first exploratory analysis was performed to test this hypothesis in order to understand whether environmental conditions varied between the burnt and unburnt sites where *Podarcis* populations were sampled. To do this, temperature and relative humidity were collected with dataloggers (Hygrochron iButton, Maxim Integrated). Given the climatic differences between locations sampled, the dataloggers were placed at the locations that are geographically closer and more probable to have similar conditions – Leonte, Lindoso and Póvoa de Lanhoso, hereafter named 'Gerês' group.

Temperature and relative humidity were registered with dataloggers during 7-days and recording information every 10 minutes in order to check if there are differences in the pattern and amplitude of both features. Two dataloggers were placed at each site under medium-size stones. So, the microhabitat where the loggers were placed did not vary between UN and BU sites; in contrast, the habitat did (forest in UN and open in BU) this fact allowing to interpret differences in terms of habitat structure between UN and BU sites. The choice of habitat had in mind the meaningful biological and ecological

features of *Podarcis guadarramae*, this is, the habitats were chosen according to the possibility of the animals actually use those structures.

After picking the dataloggers from the field, data were downloaded and the descriptive analyses (maximum, minimum, average and range per day) of records were done in STATISTICA v8 (StatSoft, 2007). In the same software, it was checked for differences between loggers in each location, in order to avoid biased results. Then, a Factorial ANOVA was performed to compare between 'Location' (Leonte, Lindoso and Póvoa de Lanhoso) and between 'Condition' (UN vs. BU) as predictive factors. When the effects of factor were significant, pairwise comparisons were examined with Scheffé's post-hoc tests.

2.5. Genetic component

2.5.1. DNA extraction

The DNA extraction was performed with the tail tip tissue of 201 sampled *Podarcis guadarramae lusitanica* lizards using EasySpin Genomic DNA Tissue Kit (Citomed, Portugal) following the manufactures' instructions. The success of extractions was evaluated by an electrophoresis in 0.8% agarose gel stained with GelRed (Biotium, U.S.A.), in TBE 0.5x (Tris-Borate-EDTA buffer: Tris 89mM, boric acid 89mM, EDTA 2mM, ph 8.0) at 300V for approximately 15 minutes. The gel was further visualized in a UV transilluminator (Bio-Rad, U.S.A.). Based on the quality and quantity of DNA in the bands observed, samples were diluted with ultrapure water in order to avoid inhibitors during the Polymerase Chain Reaction (PCR). Negative controls were used in order to detect possible contaminations. Extractions were stored at -20.0 ºC.

2.5.2. Microsatellite amplification

Microsatellites are a very popular marker for population genetics studies. Microsatellites, also known as short tandem repeats (STR), are iterations of 1-6 nucleotides motifs, with a length varying usually between five and 40 repeat units. They are found in every organism, typically at high frequencies, and are a great portion of the noncoding DNA. STRs are highly polymorphic owing to their high mutation rates, and as they are co-dominant marker they provide the ability to distinguish heterozygotes. Therefore, microsatellite markers are ideal to studying processes that act

on ecological time scales (Schlötterer, 2004; Selkoe and Toonen, 2006) and are the best option to infer modern genetic patterns (Wan *et al.* 2004).

From a battery of 26 microsatellite loci developed for *Podarcis guadarramae* (as *P. hispanica* in Agostini *et al.*, 2013), nine were chosen based on further multiplex optimization and reports of scoring errors by Ribeiro (2014). Allele scoring was based on different sizes and loci were pooled into two multiplexes previously defined by Ribeiro (2014) (**Table 3**). According to Agostini *et al.* (2013) a fluorescent dye was added to the 5'end of the forward primer of each microsatellite marker (**Table 3**).

Amplifications were performed in a final volume of 10 µL containing 5 µL of Taq PCR Master Mix (Qiagen, Germany), 3 µL of ultrapure water, 1 µL of primer mix (**Table 3**; concentrations in **Table S1**) and 1 µL of DNA template. Negative controls were used in order to detect possible contaminations. Polymerase Chain Reactions were performed in Bio-Rad T100 Thermal Cyclers (Bio-Rad, U.S.A.). Both multiplexes had equal PCR conditions. PCR cycles consisted of an initial denaturation step at 95 ºC for 15 min, followed by 15 cycles with denaturation at 95 ºC for 30 sec, touch-down annealing at 62 ºC for 1 min and 30 sec decreasing 0.5 ºC in each subsequent cycle until 55º C, extension at 72 ºC for 45 sec; 22 cycles with denaturation at 95 ºC for 30 sec, annealing at 55 ºC for 45 sec and extension at 72 ºC for 45 sec; 8 cycles with denaturation at 95 ºC for 30 sec, annealing at 53 ºC for 30 sec and extension at 72 ºC for 45 sec; ending with a final extension step at 60 ºC for 30 min (total time approximately 3 hours). Amplification success was assessed by electrophoresis in a 2.0% agarose gel stained with GelRed, and adding a DNA ladder NZYDNA Ladder V (NZYTech, Portugal). The resultant gels were visualized in an UV transilluminator. The final product was diluted if necessary.

PCR products were then separated by size on an automatic sequencer ABI 3130xl Genetic Analyser (Applied Biosystems, U.S.A.) using 1 µL of amplification product for 10 µL of formamide with size standard GS500 LIZ (Applied Biosystems, U.S.A.). Allele scoring was performed with GeneMapper v4.1 (Applied Biosystems, U.S.A.) and checked manually.

Table 2.3. List of analysed microsatellite loci and respective characterization used in this work.

Locus	**Repeat motif**	**Allele range (bp)**	**Fluorescent label**	**Multiplex**
Ph17	$(TATC)_n$	149-225	NED	C
Ph21	$(AGAT)_n$	127-189	VIC	
Ph30	$(TCTA)_n$	108-148	FAM	
Ph38	$(GATT)_n$	100-136	PET	
Ph50	$(ATGC)_n$	266-282	VIC	
Ph43	$(AGGG)_n$	136-153	FAM	E
Ph70	$(CTT)_n$	162-195	PET	
Ph81	$(TGT)_n$	288-821	FAM	
Ph128	$(GTT)_n$	218-233	VIC	

2.5.3. Microsatellite data analyses

Firstly, it was defined a threshold for allowed missing data of 20% at sample or loci level. No sample or locus were excluded from the dataset. The final dataset remained with 201 samples and nine microsatellite loci. There are three common microsatellite scoring errors that can bias ecological and evolutionary data interpretation: null alleles, large-allele dropout (or short allele dominance) and stuttering (Dewoody *et al.*, 2006). These genotyping errors can cause deviations from Hardy-Weinberg equilibrium (HWE), especially heterozygosity deficiency which can be mistakenly interpreted as inbreeding, assortive mating or Wahlund effects (van Oosterhout *et al.*, 2004). Because genotyping errors have an “allelic signature”, it is possible to distinguish whether such deviations from HWE are caused by non-panmixia or by these genotyping errors. The presence of null alleles or of scoring errors in genotyping was tested for each locus in each population sampled using MICRO-CHECKER v2.2.3 (van Oosterhout *et al.*, 2004). If the program detects the presence of null alleles, and if it is throughout the population sampled we will remove them.

For each population, deviation from HWE was assessed by a locus-by-locus exact test (analogous to Fisher’s exact test). Linkage desiquilibrium (LD) between all pairs of loci was tested based on a likelihood-ratio test using 1,000 permutations to achieve significance ($\alpha = 0.05$). These analyses were performed in ARLEQUIN v3.5 (Excoffier and Lischer, 2010). Bonferroni corrections were applied whenever multiple comparisons were made using the same procedure (Rice, 1989).

2.5.4. Genetic diversity indices and patterns of variation

Allele frequencies, expected (H_E) and observed (H_O) heterozygosities, mean number of alleles (N_A) and number of private alleles (P_A) were calculated with ARLEQUIN v3.5 (Excoffier and Lischer, 2010). The allelic richness permuted by the lowest number of individuals genotyped in a locality (A_R; Petit, 1996) and the deviation from Hardy-Weinberg proportions, F_{IS}, per population (Weir and Cockerham, 1984) were calculated for each population with FSTAT v2.9.3.2 (Goudet, 1995).

To test genetic differentiation among all populations, pairwise F_{ST} values and significance values of each pairwise comparison were calculated. An hierarchical population structure was evaluated through an analysis of molecular variance (AMOVA) in a locus-by-locus basis and integrated over all loci, based on allelic frequency data and grouping by location (i.e. 5 groups) in order to test the null hypothesis that genetic variation was not associated with spatial structure according to the five locations. Both analysis were done in ARLEQUIN v3.5 (Excoffier and Lischer, 2010) with significance ($\alpha = 0.05$) was achieved using 10,000 permutations.

A Principal Coordinate Analysis (PCoA) based on Nei's standard genetic distance (D_{ST}; Nei, 1972) was performed in GenAlex v6.5 (Peakall and Smouse, 2012) in order to visualize patterns of genetic differentiation among populations.

To visualize spatial patterns of genetic distances (i.e. discontinuities among populations) over the study area, a genetic landscape shape was constructed with the program Alleles in Space v1.0 (Miller, 2005). A Delaunay triangulation connectivity network (Watson, 1992; Brouns *et al.*, 2003) was created based on each sampling location and assigns a genetic distance at the geographic midpoint of each population pair. The lizards of a population have the same spatial coordinates which reflects the population-based sampling strategy of this study, however average between location inter-individual genetic distances are used to infer putative barriers or calculate surface heights (Miller, 2005). The software uses a simple genetic distance that quantifies the proportion of genotypic differences between individuals from different populations. An interpolation procedure (inverse distance-weight, *a*) was used to produce a three-dimensional surface plot where *X*- and *Y*-axes correspond to population geographical coordinates and surface plot heights (*Z*) represent interpolated genetic distances. Analysis was performed with a grid size of 100 × 100 and a range of interpolation parameters ($a = 0.2–1.0$). Because greater values of *a* caused interpolated values to be more influenced by close points, the analyses were carried out for a distance weighting parameter of $a = 0.2$. Following the Delaunay triangulation network constructed before, the Monmonier Maximum Difference Algorithm (Monmonier, 1973) was used to build

putative genetic barriers across the landscape. The first step is to identify the greatest genetic distance between any two locations joined in the connectivity network, i.e. the initial barrier segment. Then, the initial barrier is followed in one direction until it encounters either an external edge of the connectivity network or an internal segment previously defined as a barrier segment. Basically, for each extension of the barrier, the movement is in the direction of the highest genetic distance between locations. Finally, the initial barrier identified is followed in the opposite direction to the second barrier, until, once again, encountering either an external edge of the connectivity network or an internal segment previously defined as a barrier segment.

A Bayesian clustering approach implemented in STRUCTURE v2.3.4 (Pritchard *et al.*, 2000) was used to detect a genotype-based refined structure (other than the pairwise F_{ST}, which is allele-frequency-based, and PCoA) in the dataset. It is a model-based method which assumptions are HWE within populations and can assume both complete and incomplete linkage equilibrium between loci within populations. Based on the set of allele frequencies of each cluster detected, each sampled individual is probabilistically assigned to a certain group based on the proportion of genome identity. Two exploratory analyses were performed: first using the entire dataset with no prior population information; and second, using the populations sampled as putative origin populations of individuals. It was further investigated if there was substructure in each population pair. For both proceedings, it was used the admixture model with correlated allele frequencies with 10 independent replicates consisting of 250,000 MCMC iterations after a burn-in of 100,000 steps. When the entire dataset was used the number of clusters, *K*, ranged from 1 to 10, whereas when only the pairs of populations were tested *K* varied between 1 and 2. The results were uploaded to STRUCTURE HARVESTER (Earl *et al.*, 2012) to identify the most likely number of *K* that best explained genetic structure in the entire dataset and within pairs of populations. Both the highest posterior probability, Ln Pr ($X|K$), (Pritchard *et al.* 2000) and the Evanno's method of Delta *K*, ΔK, based on the second-order rate of change in the log probabilities between successive values of *K* (Evanno *et al.*, 2005) were used to assign the most probable number of populations.

2.5.5. Genetic differentiation and fire-history parameters

We compared some diversity indices, N_A, A_R, P_A, H_E, and the coefficient F_{IS} were compared between UN and BU populations of each site with scripts written in Python 2.7.1 (available from C. Pinho upon request). The same analyses were performed pooling all UN populations and compare it with the pool of BU populations. The significance ($\alpha = 0.05$) was achieved by 999 permutations.

As stated in the Experimental design section, the populations sampled form a gradient regarding the fire-history (number of fires, time since the last fire) and some analyses would be more valuable to infer patterns by disregarding the paired sampling scheme and only use the UN and BU classes. Therefore, the same genetic measures (N_A, A_R, H_E, F_{IS}) of all population (thus, disregarding the paired design) were correlated with respective fire factors (number of fires and time since last fire) using simple linear regressions in STATISTICA v8.0 (StatSfot, 2007).

To identify which are the most likely causes of the differentiation patterns, it was tested the effect of geographic distance as well as the fire-history on the observed genetic differentiation. Pairwise genetics distances between populations were computed by the Nei's standard genetic distance (D_{ST}; Nei, 1972) as implemented in GenAlex v6.5 (Peakall and Smouse, 2012). Geographic distances between populations were calculated from the pairwise Euclidean distance according to the latitude and longitude of the location of each population with Geographic Distance Matrix Generator, version 1.2.3 (Ersts, American Museum of Natural History, Centre for Biodiversity and Conservation,
http://biodiversityinformatics.amnh.org/open_source/gdmg). To construct the fire-history of each population, a table containing the presence (listed as 1) or the absence (listed as 0) of fire in each year from 1975 to 2013 was produced. From this table, we compared differences in fire-history patterns between pairs of populations with the Jaccard index (**Table S2**) implemented in the software PRIMER v5 (Clarke and Gorley, 2001). Thus, a matrix based on the pairwise differences between populations regarding their fire-history was constructed. It was used Mantel tests (Mantel, 1967) to test for an association between genetic and geographic distances and/or fire-history similarities. Also, partial Mantel tests (Smouse *et al.*, 1986) were used to assess the association between genetic distance and fire-history while controlling for the influence of Euclidean geographic distances. Simple and Partial Mantel tests were performed in PASSaGE v2 (Rosenberg and Anderson, 2011) and their statistical significance ($\alpha = 0.05$) achieved with 10,000 permutations.

2.5.6. Recent demographic history

All 10 populations were tested for signatures of genetic bottlenecks in order to detect a decline in the effective population size (N_E) in "recent" history (0.2 - $4N_E$ generations before present; Luikart and Cournuet, 1998) with BOTTLENECK v1.2 (Piry *et al.*, 1999). This software sets on the "heterozygosity-excess" test developed by Cornuet and Luikart (1996). Populations that experienced a recent reduction of N_E show a faster reduction of allele number than the reduction of heterozygosity under HWE (sense of Nei's, 1987). As a consequence, the expected heterozygosity under HWE becomes larger than the expected heterozygosity under mutation-drift equilibrium since the latter is calculated from the allele number. Thus, the BOTTLENECK program is used to detect a heterozygosity excess as a consequence of a genetic bottleneck, under the assumption of population mutation-drift equilibrium before the bottleneck. Another assumption required in a genetic bottleneck test is to define a microsatellite evolution model in order to generate expected distributions for test statistics (Peery *et al.*, 2012). The mutation model of microsatellites on *Podarcis* show irregular mutation patterns, this is, it does not strictly follow any of the mutation models. More likely, it is more proximate to the infinite alleles model (IAM) than the other two, stepwise mutation model (SMM) and the intermediate two-phase model (TPM, Di Rienzo *et al.*, 1994), yet, the bottleneck tests were performed for those three mutation models incorporated in the program. The TPM was tested with the intermediate value at 50% of multistep mutations in each analysis, with a variance of 12 (Piry *et al.*, 1999).This percentage makes it the exact intermediate mutation model between the extremes IAM and SMM. All analyses were ran with 10,000 iterations. Significance of heterozygosity excess in each of the locus and averaged across loci was determined with one-tailed Wilcoxon test under the null hypothesis of no significant heterozygosity excess (Piry *et al.*, 1999). BOTTLENECK also tests for allele frequency mode-shifts, this is, the distortion away from a normal L-shaped allele frequency distribution expected under mutation-drift equilibrium, when rare alleles (i.e. <0.1%) are numerous.

The effective population size of each population, N_E, was estimated as well as the migration rates between the UN and BU populations at each location with Bayesian coalescent-based methods implemented in MIGRATE-N v3.6 (Beerli and Felsenstein, 2001). We are testing the hypothesis that fire caused a decrease in the effective population size (either by mortality or by indirect effects such as habitat destruction), and if maybe the fire would impose a migration pattern in a specific direction (higher gene flow towards UN or BU populations). This software assumes that populations have stable population sizes and migration rates for a long time (~4N generations), which might

hamper the correct estimation of N_E in some populations (e.g. that experienced previous bottleneck episodes) and therefore warrant is necessary when analysing the results to avoid misleading interpretations. The mutation-scaled effective population size, Theta, $\Theta = 4N_E\mu$, where μ is the mutation rate per generation per locus, and the multi-scaled migration rates $M = m/\mu$, where m is the migration rate per generation among populations, were calculated.

Most MIGRATE-N parameters were left at default values, but adjustments were made on parameters influencing the run-length, relative mutation rate, and to specify different migration models. Given the low differentiation between populations (small values of F_{ST}; **Table 6**) it was performed a full migration model with variable Theta (Θ) and allowing all populations to have different sizes and migration rates. A random genealogy and parameter (Θ and M) settings inferred by an F_{ST}-based method where used as start condition. The mutation rate among loci was scaled so that the average rate change of the mutation rate was 1.0. This equalizes the effects of the estimates of the individual loci. The relative mutation rate among loci estimated from the data varied from 0.5 at locus Ph43 to 1.5 at Ph21. The Markov Chain Monte Carlo settings were as follows: the first 500,000 steps were discarded, then 50,000 samples were recorded every 50th step, using parallel runs of 4 replicates. A step comprises of either a parameter change or a genealogy change. A total of eight loci yielded samples of 20 million steps. The prior distribution for the parameters was uniform with boundaries appropriate for the parameters and data (microsatellites): Theta priors were bounded between 0 and 100 and M priors where bounded between 0 and 1000.

2.6. Morphological component

Several studies have shown that developmental instability is a useful measure of environmental stress in native populations (e.g. Palmer and Strobeck, 1986; Leary and Allendorf, 1989; Parsons, 1990). Therefore, we tested if populations with different condition (i.e. being UN or BU) showed signs of developmental instability as a morphological response the multiple fires through environmental and/or genetic stress.

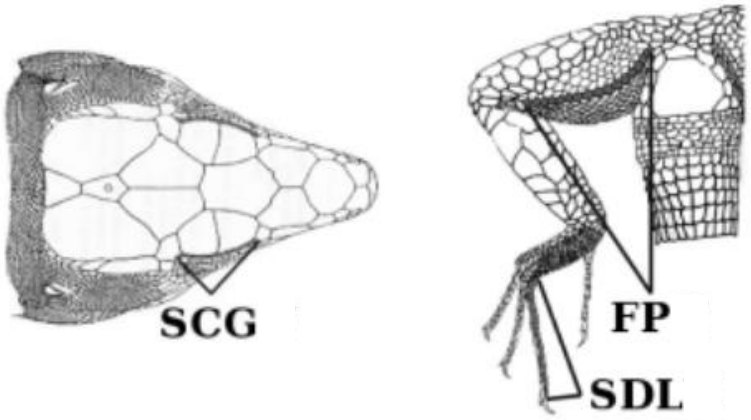
To test this hypothesis, an analysis to the bilateral symmetry of individuals of each population was performed, this is, a search for random deviations from perfect symmetry of each trait (null hypothesis). If there is some signal of deviation, it would be tested if those patterns were correlated with the condition of each population (being UN or BU) – since one of this work's hypothesis is that a repeated fire regime affects populations at both the genetic and morphological level. It was expected that given the environmental harshness of burnt areas (e.g. higher temperature as well as lower humidity, both with

higher amplitude), burnt lizard populations would show higher degree of deviation of symmetry. The methodology was done following Lazić *et al.* (2013), in which they evaluated how the developmental stability of populations is influenced by pollution using an analysis of asymmetry to quantify the effect of such environmental disturbance. These authors used two types of populations: rural (control populations - low level of pollution) and urban (high levels of pollution) populations. In our case, instead of pollution the perturbation is the environmental harshness (higher temperatures, lower humidity) imposed by the occurrence of repeated fires.

2.6.1. Choosing the right traits

Upon sampling, pictures of three meristic characters were taken for each lizard: femoral pores (FP), subdigital lamellae (SDL) on the 4th toe of each hindlimb, and supracilliar granules (SCG). Meristic traits were chosen because they can be measured with little or no error (Palmer, 1994). Moreover, with this combination of traits we can test for differences across functional traits (i.e. FP and SDL are functional traits such reproduction and hiking capabilities respectively, and can also be a surrogate for limb's length) and non-functional ones (i.e. the SCG have, supposedly, no function). Specifically, these traits were selected because they are easy and fast to quantify and present extensive variability in *Podarcis* populations and species (Kaliontzopolou *et al.*, 2012b). They already showed to be experimentaly sensitive to disturbances such as in the case of Lazić *et al.* (2013) where they studied the developmental instability of *P. muralis* due to environmental stress, namely pollution; or the case of *Lacerta agilis* in Zhadanova and Zakharov (2006) where they saw that hatchlings incubated under or above optimum temperature had more fluctuating asymmetry.

Figure 2.7. Morphological traits used for the analysis. SCG – supracilliar granules; FP – femoral pores; and SDL – subdigital lamellae. Image taken from Kaliontzopoulou *et al*. (2011).



Each trait was photographed at both sides of the body with a Sony PowerShot (resolution 10 MP). Femoral pores and subdigital lamellae are functional traits (FP, chemical signalling; SDL, adhering and climbing surfaces) and most likely reflect the length of the limb, whereas the supracilliar granules have no evident function. By saying this, it was expected that functional traits would show less deviation than the traits that have no apparent adaptive function. Each trait was characterized by the number of scales present. The counting of scales was done twice in order to be able to account for the measurement error. Moreover, both counts were separated by a reasonable amount of time, and the counting was done in a random manner between individuals in order to ensure the independence of trait count.

2.6.2. Analysis of symmetry

First, an asymmetry index (AI) was calculated for each of the three traits as the number of scales on the right side minus that on the left side (AI = R–L). Some exploratory analyses were done before proceeding to the detection of asymmetry:

1) a test for normality. The AI index was tested for normality with Kolmogorov-Smirnov test within each population in all traits. All populations showed that all the analysed traits followed a normal distribution ($p > 0.05$).

2) a test for sexual dimorphism in the traits examined. To proceed with the analysis in a more straight-forward manner it was important to test for the presence of sexual dimorphism in relation to the number of scales of each trait (i.e. if there are not variation between sexes, both genders can be analysed altogether). For that it was performed an independent t-test by population with the AI of each trait as the dependent variable and sex as the grouping variable. There was no sexual dimorphism in the traits examined in any population ($p > 0.05$) and therefore further analyses were done with all the individuals sampled independently of the gender.

3) a test to detect if the asymmetry was dependent on trait size. Because traits with higher number of scale are more prone to show higher AI, trait size dependence was tested for each trait separately by a linear regression of the unsigned AI values (|AI|) on the average between sides [(R+L)/2] of the respective trait. It was not detected a correlation between asymmetry and trait size (see Results).

In order to test for the presence of deviations to bilateral symmetry which can be directional (DA) or fluctuating asymmetry (FA), a two-way ANOVA was performed while taking into account the measurement error. The ANOVA was done separately for each population and for each trait with its respective log-transformed trait values (i.e. number

of scales) and using 'Side' as fixed factor, 'Individual' as random factor, as well as their interaction. If only the effect of side is significant, it would mean that the deviation is biased towards one of the sides (right or left) and the asymmetry is directional (DA). In contrast, if the interaction between 'Side' and 'Individual' has a significant effect, then it means that there is variation from individual to individual relative to the side where deviation arises and we are in the presence of fluctuating asymmetry.

Since the results point to the existence of fluctuating asymmetry (FA) in all populations for all traits (see Results), an individual asymmetry index (i.e. individual FA index) was calculated for each trait based on the difference between the log-transformed average between the two counts to account for measurement error [|ln (R average) – ln (L average)|]. Then, with a four-way ANOVA it was tested for the effects of condition (UN vs. BU), populations (nested within condition), sex and trait on the individual FA index. All interactions effects were also examined. This would show if the observed level of FA is related to the population examined (each population could have a different pattern), or the type of population (UN vs. BU), or just an effect of the different asymmetry levels of each trait, the same for sex.

Since the development of all traits are exposed to the same level of disturbance, it was tested: i) if all traits had the same degree of asymmetry, and ii) if traits of the same body part had similar degree of asymmetry (e.g. if femoral pores and subdigital lamellae in the right side are more symmetric). Therefore, it was tested whether asymmetry was organism-wide by examining the correlation between traits in unsigned FA (|R-L|). If the correlation is significant it means that if a particular trait shows asymmetry then the trait that it showed a significant correlation with, would be asymmetric as well. And so the FA in a single trait can be used as an indicator of individual quality. It was also examined the correlation between traits in signed FA (R–L) (using the signed measure allows to differentiate the direction of the asymmetry, right or left) to test if traits developing in the same body part show similar asymmetry patterns.

Given the results of the previous analysis (see Results), the four-way ANOVA was repeated only with the Gerês group (Leonte, Lindoso and Póvoa de Lanhoso) since the closer geographic distance makes them 'perfect' replicates as they are, supposedly under the same environmental conditions (i.e. similar latitude, distance to the sea and altitude). The trait size dependence was examined again in each trait separately by a linear regression of the (|AI|) values on the average between sides [(R+L)/2] of the respective trait. And finally, the correlation between traits in unsigned FA (|R-L|) and between traits in signed FA (R–L) was done.

All the analyses were done in STATISTICA v8 (StatSoft, 2007).

2.7. Genetic component and asymmetry

One of the mechanisms explaining the maintenance of development stability can have a genome-wide basis (Clarke, 1998): the Heterozygosity Theory, which says that genetic diversity have a buffering role by increasing biochemical diversity and thus enabling a dynamic stable developmental pathway in changing environments. Likewise, developmental instability can be enhanced by a decreased ability to cope with environmental changes due to low genetic diversity (e.g. inbreeding and genetic drift).

Given the importance of the genetic basis on the stable development of individuals (Heterozygosity Theory; Clarke, 1998), an individual multilocus heterozygosity (IMH) measure was calculated. This measure referrers to the proportion of typed loci that were heterozygous in each individual having into account the number of alleles present in the locus. The IMH was tested for a correlation with the individual FA index (see previous section for more information) of each trait and the average across all traits with Linear regression in STATISTICA v8 (StatSoft, 2007).

Moreover, a population FA index was calculated with the averaged FA value across the individuals of each population, for each trait and for the average value across the three traits. Then it was further tested a correlation of population FA index (with each trait separately and with the averaged value across them) with N_A, A_R, H_E, and F_{IS} of populations.

3. Results

Content

3.1. Environmental conditions

The ANOVA performed to compare the descriptive statistics of temperature between locations and between conditions showed that there were significant differences in both factors (**Table 4; Table 5**). Burnt environments achieved higher mean, maximum and range temperatures (post-hoc Scheffé, $p < 0.05$; **Fig. 10**). But locations are also significantly different in all statistics except for the range (see **Table 4**), which means that although the significant differences in mean, maximum and minimum, the range does not differ. In contrast, only the humidity range was significantly different between conditions ($F = 17{,}768$; $p = 0.0056$), in which burnt environments had a significant higher range (post-hoc Scheffé, $p < 0.05$; **Fig. 11**). These results showed that the burnt habitats receive more radiation and have higher amplitude of day-night temperatures, consequently higher fluctuation in the relative humidity was observed in burnt populations.

Table 3.1. Summary results of ANOVA on the descriptive statistics of temperature (T) of the different locations (Leonte, Lindoso, Póvoa de Lanhoso) and for both populations (UN and BU) where dataloggers were placed. *df* – degrees of freedom; *F* – F-statistics; *p* – *p* value.

		Mean T		**Max. T**		**Min. T**		**T range**	
	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*P*
Loc.	2	21,485	**0,0018**	14,538	**0,0050**	5,959	**0,0375**	1,168	0,3729
Cond.	1	10,096	**0,0191**	19,890	**0,0043**	0,242	0,6400	10,066	**0,0193**
Loc.*Cond.	2	1,306	0,3381	0,969	0,4320	0,207	0,8188	0,140	0,8721

Table 3.2. Summary results of ANOVA on the descriptive statistics of humidity (Hum) of the different locations (Leonte, Lindoso, Póvoa de Lanhoso) and for both populations (UN and BU) where dataloggers were placed. *df* – degrees of freedom; *F* – F-statistics; *p* – *p* value.

		Mean Hum		**Max. Hum**		**Min. Hum**		**Hum range**	
	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Loc.	2	2,521	0,1604	1,539	0,2887	2,733	0,1433	2,001	0,2159
Cond.	1	0,847	0,3930	0,000	1,0000	2,187	0,1897	17,768	**0,0056**
Loc.*Cond.	2	0,355	0,7148	0,492	0,6340	0,095	0,9111	1,665	0,2662

Figure 3.1. Differences between the burnt (BU) and unburnt populations (UN) for the descriptive statistics of temperature. There was significantly higher values in BU than in UN represented by the asterics (*) in the respective measure. * - significance value of Scheffé post-hoc tests between UN and BU.

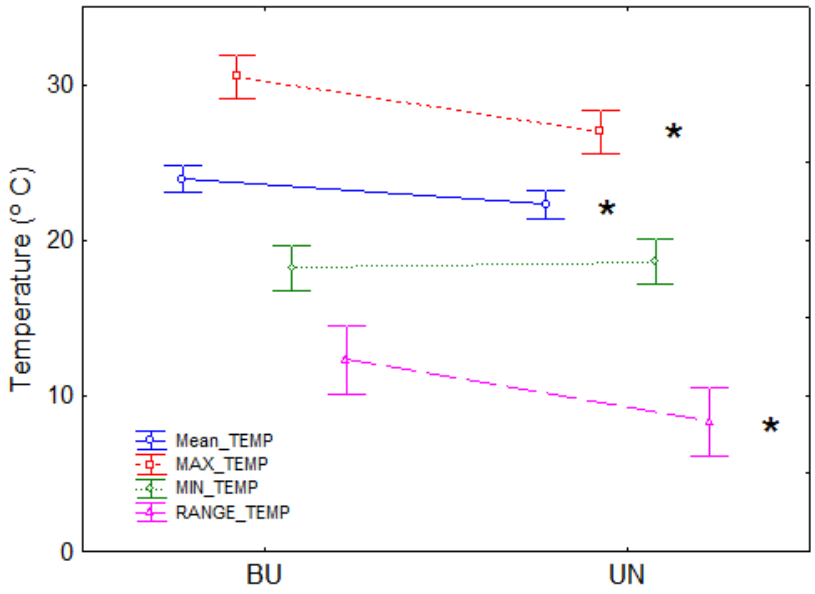
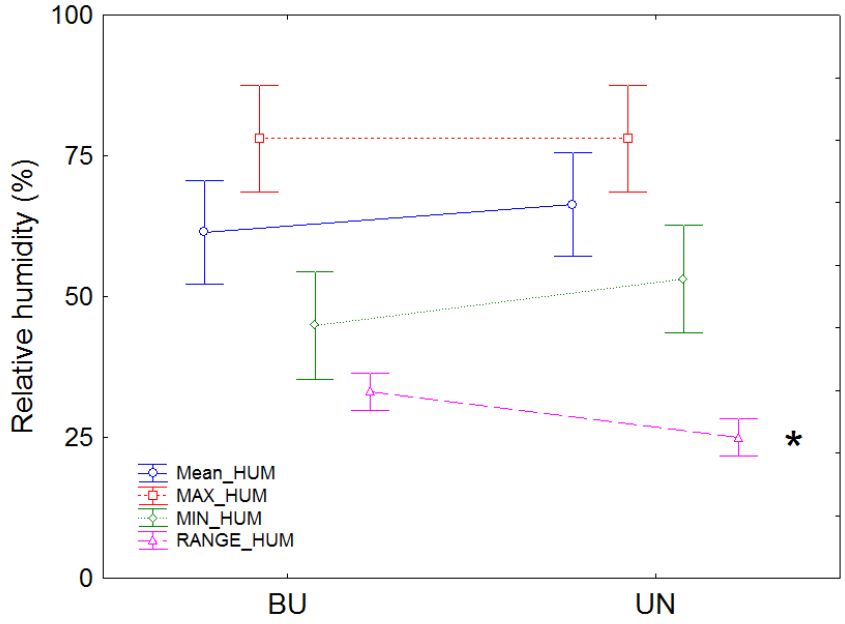


Figure 3.2. Differences between the burnt (BU) and unburnt populations (UN) for the descriptive statistics of humidity. There was significantly higher values of humidity range in BU than in UN represented by the asterics (*) in the respective measure. * - significante value of Scheffé post-hoc tests between UN and BU.



3.2. Genetic component

3.2.1. Genotyping errors and deviations from equilibrium

From the initial battery of nine microsatellite loci genotyped, one (Ph17) showed presence of null alleles in seven out of 10 populations (**Table S3**) and for this reason it was excluded from further analyses. The remaining eight loci were kept although there were five loci presenting null alleles and one locus of them had also stuttering (**Table S3**), since it was not genome or population wide, it was most likely an artefact derived from the sampling scheme. Further results of HWE and LE were then analysed having this into account, and if those loci showed deviations from equilibrium on the light of existence of null alleles they would be excluded. No deviations from either HWE or LE were found in the eight loci that composed the final dataset. We removed Ph17 from the dataset due to null alleles in several populations (**Table S3**).

3.2.2. Genetic diversity patterns and population structure

The number of alleles per locus varied between 5 (Ph128) and 19 (Ph21). Overall, the average heterozygosities (H_E) of the eight loci genotyped showed moderate values, ranging from 0.663 to 0.732 at Póvoa de Lanhoso UN and Santo Tirso BU, respectively (**Table 6**). The mean number of alleles (N_A) per population varied from 6.125 at Póvoa de Lanhoso UN to 8.625 at Moledo BU, and accordingly, the allelic richness (A_R) was lower and higher at the same locations with 5.922 and 7.763, respectively. Considering all populations sampled, four of them had private alleles and were mostly populations from burned areas (see **Table 6**), with exception to Moledo UN. Some diversity indices, such as the N_A, A_R and H_E showed a general pattern of increasing divert values from UN populations towards BU populations, except from Leonte that showed the contrary pattern (see **Table 6**). The H_O did not show a clear pattern across the locations, three out of five showed decreasing values towards BU populations. Among BU populations, Leonte BU showed the lowest allelic richness and expected heterozygosity and equal mean number of alleles compared to its relative UN population. There was a trend of increasing F_{IS} towards the BU populations detected for all pairs, where F_{IS} is lowest at Leonte UN (0.004) and highest at Póvoa de Lanhoso BU (0.170).

Table 3.3. Summary of the genetic diversity indices calculated for each of the 10 populations sampled. UN – unburnt; BU – burnt; N – number of individuals sampled; N_A – mean number of alleles; A_R – allelic richness; PA – number of private alleles; A_R – allelic richness; P_A – number of private alleles in a population having into account all the sampled populations; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – deviation from HW proportions and bold values are those that are statistically different from zero (Weir and Cockerham, 1984).

Population	N	N_A	A_R	P_A	H_O	H_E	F_{IS}
Leonte UN	21	7.125	6.786	-	0.718	0.703	0.004
Leonte BU	20	7.125	6.736	-	0.608	0.686	**0.139**
Lindoso UN	20	6.750	6.474	-	0.619	0.677	**0.112**
Lindoso BU	17	7.250	7.152	-	0.621	0.677	0.114
Santo Tirso UN	20	7.375	6.961	-	0.659	0.719	**0.109**
Santo Tirso BU	21	7.750	7.327	1	0.648	0.732	**0.140**
Moledo UN	20	6.875	6.507	1	0.665	0.672	**0.036**
Moledo BU	22	8.625	7.763	3	0.669	0.693	**0.057**
Póvoa de Lanhoso UN	20	6.125	5.922	-	0.656	0.663	0.036
Póvoa de Lanhoso BU	20	7.750	7.251	5	0.613	0.712	0.170

Almost all population pairs showed significant, although low, allele-frequency-based differentiation (**Table 7**). Significant pairwise F_{ST} values were low (0.015-0.088) (based on Wright, 1978), being the lowest value between Leonte UN and Lindoso UN, and the highest corresponds to the pair Moledo UN and Lindoso BU. With exception of Moledo, all remaining locations showed no significant differentiation regarding the pairs of populations. Obviously, the pairs that form the Gerês group (Leonte, Lindoso and Póvoa de Lanhoso) had lower values of differentiation ranging from 0.0003 to 0.0342.

The results of the AMOVA revealed that all levels of genetic differentiation were significant (see **Table 8**). Most of the genetic variation was found to be related within populations (96.2%) and only a small, but significant, percentage attributed to the variation among locations (2.17%) and to among populations of each location (1.63%), which corroborates the fact that almost all pairwise F_{ST} values were significant.

Table 3.4. Summary results of analysis of molecular variance (AMOVA) within and among locations sampled. SS – Sum of squares; VC – Variance component; * p = 0.003; ** p = 0.000.

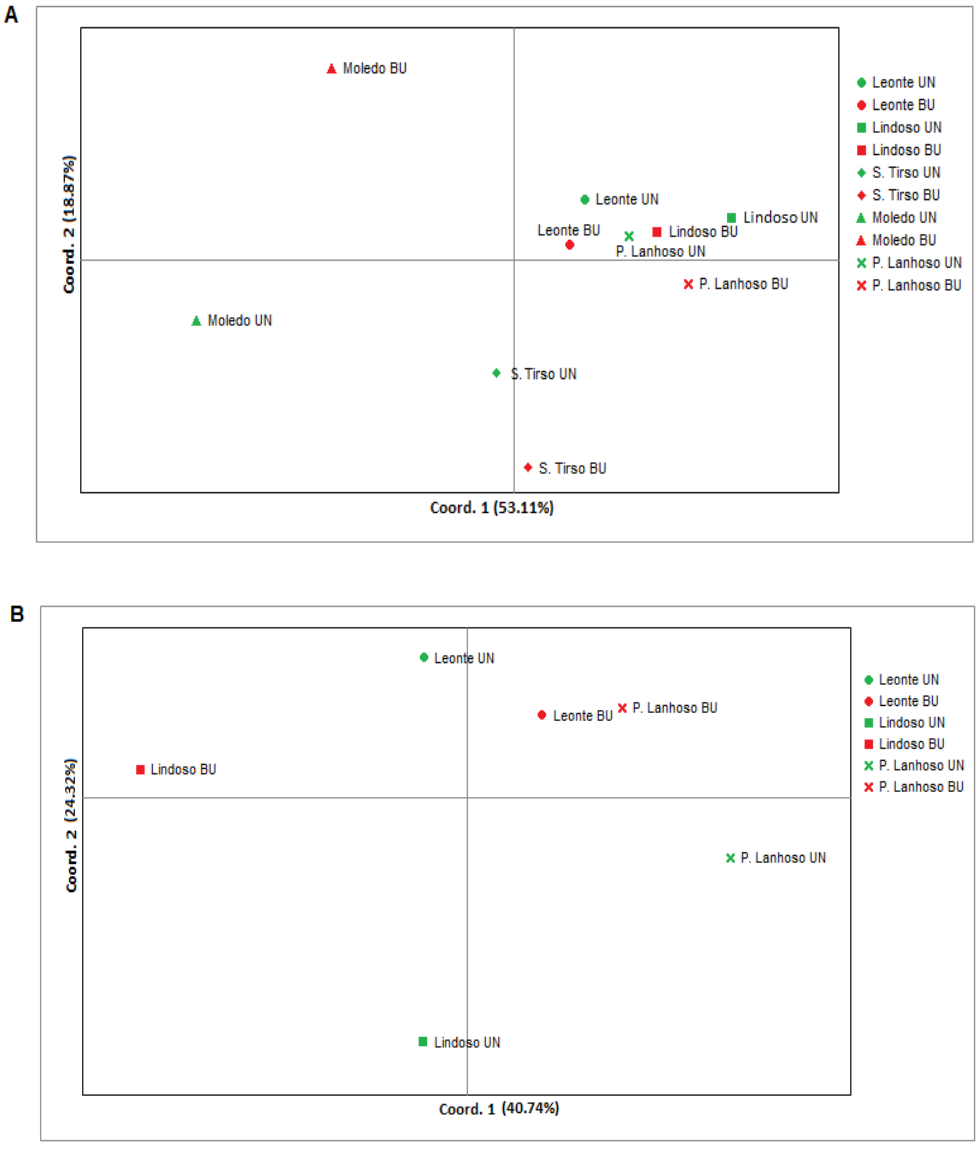
Source	SS	VC	Variation (%)	Fixation indices	
Among location	39.429	0.06429	2.17087	F_{ST}	0.03796**
Among populations within location	23.851	0.04812	1.62500	F_{SC}	0.01661*
Within populations	1106.559	2.84894	96.20413	F_{CT}	0.02171**
Total	1169.839	2.96135			

Table 3.5. Measures of pairwise differentiation for 10 populations sampled based on F_{ST} (below the diagonal; bold values are significant) and significance values with standard deviation between braquets (above diagonal). UN – unburnt; BU – burnt.

	Leonte UN	**Leonte BU**	**Lindoso UN**	**Lindoso BU**	**S.Tirso UN**	**S. Tirso BU**	**Moledo UN**	**Moledo BU**	**P. Lanhoso UN**	**P. Lanhoso BU**
Leonte UN	-	0.0003	**0.0148**	**0.0184**	**0.0179**	**0.0256**	**0.0522**	**0.0315**	0.0098	0.0089
Leonte BU	0.5456 (0.0149)	-	**0.0192**	0.0178	0.0160	**0.0226**	**0.0520**	**0.0376**	**0.0246**	0.0130
Lindoso UN	0.0410 (0.0065)	0.0391 (0.0063)	-	**0.0219**	**0.0392**	**0.0413**	**0.0735**	**0.0657**	**0.0227**	**0.0218**
Lindoso BU	0.0225 (0.0044)	0.0635 (0.0090)	0.0264 (0.0048)	-	**0.0439**	**0.0445**	**0.0877**	**0.0715**	**0.0342**	**0.0216**
S. Tirso UN	0.0147 (0.0034)	0.0606 (0.0067)	0.0010 (0.0010)	0.0000 (0.0000)	-	0.0013	**0.0456**	**0.0401**	**0.0360**	**0.0224**
S. Tirso BU	0.0010 (0.0010)	0.0186 (0.0036)	0.0000 (0.0000)	0.0000 (0.0000)	0.4981 (0.0160)	-	**0.0445**	**0.0541**	**0.0228**	**0.0202**
Moledo UN	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)	-	**0.0467**	**0.0770**	**0.0744**
Moledo BU	0.0010 (0.0010)	0.0010 (0.0010)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)	0.0010 (0.0010)	-	**0.0489**	**0.0552**
P. Lanhoso UN	0.0889 (0.0091)	0.0068 (0.0030)	0.0098 (0.0029)	0.0029 (0.0016)	0.0010 (0.0010)	0.0313 (0.0046)	0.0000 (0.0000)	0.0000 (0.0000)	-	0.0105
P. Lanhoso BU	0.1670 (0.0097)	0.1084 (0.0097)	0.0254 (0.0040)	0.0293 (0.0050)	0.0166 (0.0039)	0.0166 (0.0042)	0.0000 (0.0000)	0.0000 (0.0000)	0.1416 (0.0119)	-

The PCoA obtained through Nei's genetic distance (**Table S4**) suggested a partial paired-organized structure, although with considerable genetic distance between population pairs in Moledo and Santo Tirso, while Leonte, Lindoso and Póvoa de Lanhoso are genetically more similar to each other than to the other two locations, as expected for the geographic distribution (**Fig. 12-A**). The two coordinate axes together explained 71.98% of the total genetic variation. The "cluster" observed in **Fig. 12-A** is formed by the populations that are geographically closer, namely the Gerês group. A second PCoA only with the six populations of the Gerês interestingly identified Leonte UN as more similar to the BU populations that to others UN in the second PCoA axis (**Fig. 12-B**). In this case, the two coordinate axes explained 65.06% of the total genetic variation.

Figure 3.3. Patterns of genetic variation plotted with a Principal Coordinate Analysis based on the standard genetic distance (D_{ST}; Nei,1972). A) all the 10 populations sampled; and B) populations of Gerês group formed by the pairs of Leonte, Lindoso and Póvoa de Lanhoso.



Overall, the Genetic Landscape Shape interpolation analysis produced surface plots of genetic structure and variation along an East/West axis, in which genetic distances decreased towards populations located more Northeastern of the study area (**Fig. 13**). The observable 'ridges' are West orientated, which indicate greatest genetic distances: the highest corresponds to the population in the Northwest extreme of the study area – Moledo BU; the second highest pick is more to southeast and corresponds to Póvoa de Lanhoso BU; a third much smaller ridge corresponds to the middle latitude of the study area – Póvoa de Lanhoso UN; the fourth ridge corresponds to the most southernmost population which is Santo Tirso location. Moreover, the area inbetween these ridges presents a smooth surface, indicative of low differentiation although with small fluctuations. The software detected two barriers (**Fig. 14**), the first separates Moledo populations from the remaining ones while the second separates Santo Tirso populations from the rest, just as in the PCoA (**Fig. 12-A**).

Figure 3.4. Results of a Genetic Landscape Shape interpolation analysis for *P. guadarramae*. It used a 100 x 100 grid and a distance weighting parameter (a) of 0.2, with the map of the study area. X- and Y-axes correspond to geographic locations within a Delaunay triangulation network constructed among the sampled locations. Surface plot heights reflects average between interindividual mean genetic distances in each location.

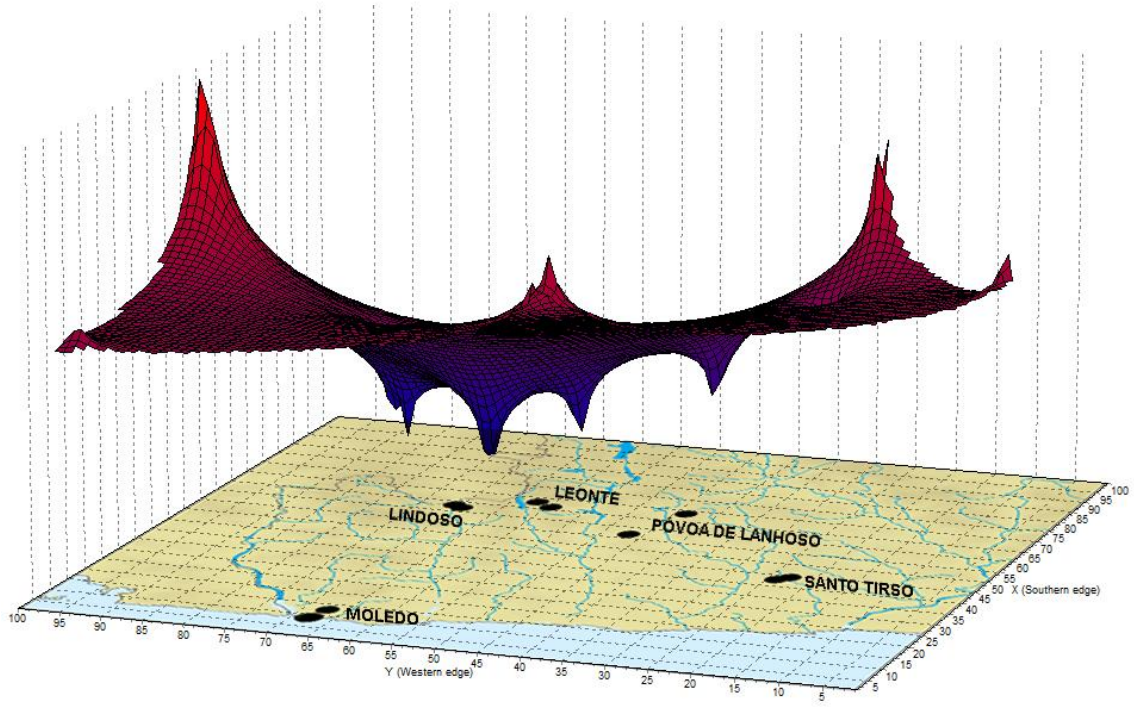
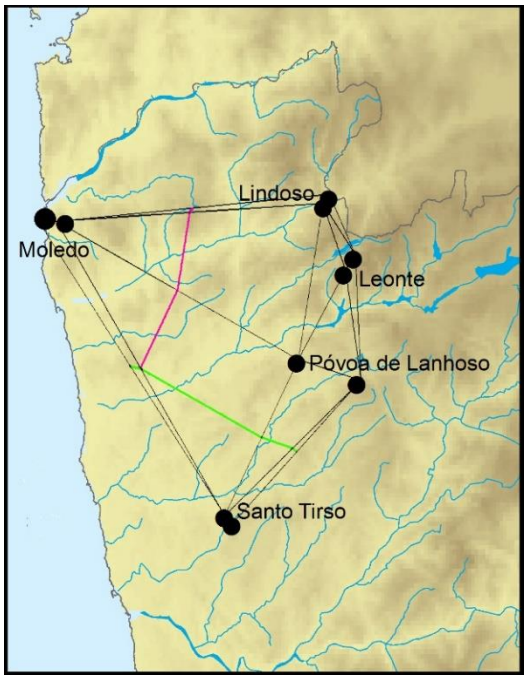
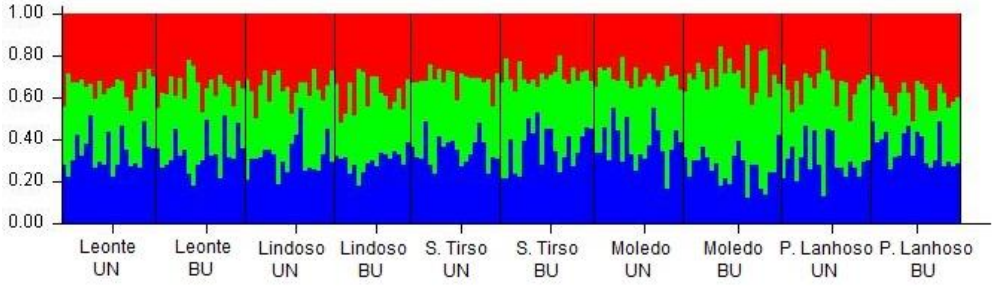


Figure 3.5. Putative genetic barriers across the landscape determined by Monmonier Maximum Difference Algorithm.



The Bayesian approach implemented in STRUCTURE v2.3.4 (Pritchard *et al.*, 2000) did not detect any structure among populations and are consistent with AMOVA results, given the low F_{ST} values between populations. On one hand, according to the highest value of Ln Pr (*X*|*K*) (Pritchard *et al.*, 2000) the best *K* was 1, this is, a single genetic cluster including all populations could be identified. On the other hand, by the Evanno's method of Δ*K* (Evanno *et al.*, 2005) the best *K* was 3, however when *K*=3 was plotted, approximately equal probabilities of individual assignment to in any of the 3 clusters were found (**Fig. 15**). This was also true for the separate analysis of each pair: Ln Pr (*X*|*K*) pointed to a *K*=1 while the Δ*K* suggested *K*=2. The same constraint was found when plotting *K*=2 (results not shown).

Figure 3.6. Model-based multilocus genotype analyses performed in STRUCTURE for *K* = 3. Each bar represent an individual.



3.2.3. Genetic diversity, geographic distances and fire-history

Apart from the absence of clear structure given by the STRUCTURE, previous results (pairwise F_{ST}, AMOVA, PCoA; see section 3.1.2.) suggest that there are differences between the populations sampled. Therefore, it would be necessary to test if the populations of each pair were statistically different and whether the observable patterns of differentiation were related to the fire-history or a result of spatial structure.

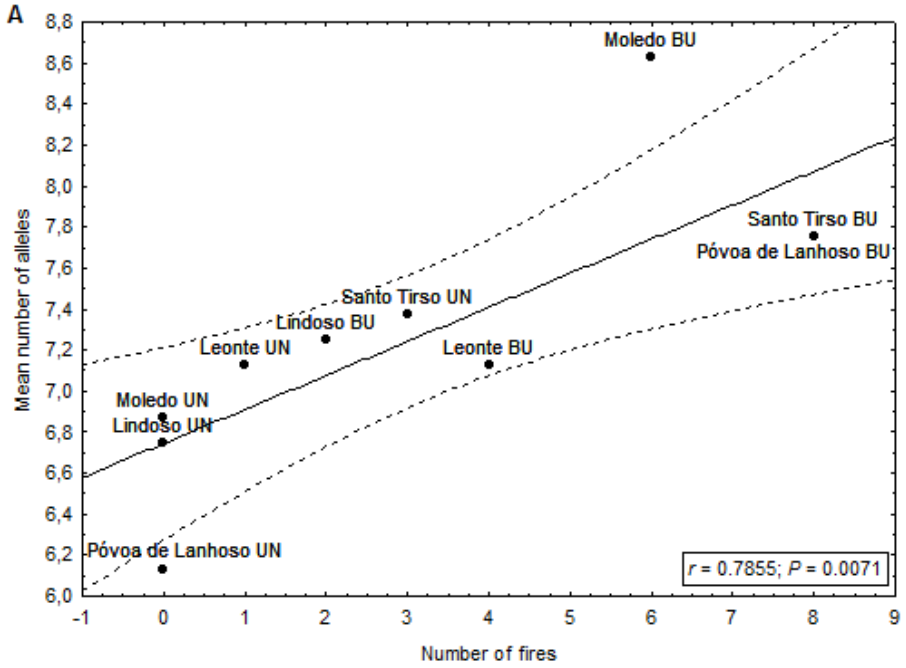
Based on the experimental design, i.e. the comparisons of populations with a different type of condition (UN and BU), each pair was tested for significant differences in the standard diversity indices: mean number of alleles (N_A), number of private alleles (P_A), expected heterozygosity (H_E) and the average deviation from HW proportions (F_{IS}). A significant difference was only reported for three out of five pairs (see **Table 9**). Leonte populations did not show any significant difference between BU and UN for any diversity measure. The same result of non-significance was found for the Santo Tirso pair. Lindoso and Moledo pairs showed the same pattern with only the N_A and P_A significantly differing between UN and BU populations although both significance values for Lindoso being marginal (P = 0.047; **Table 9**). The only pair that showed significant differences for all the diversity indices was Póvoa de Lanhoso (see **Table 9**). When comparing pools of populations with the same condition (UN vs. BU), a clear difference is reported for the N_A and P_A (see **Table 9**). Also, when examining the Gerês group composed by closer populations geographically (Leonte, Lindoso and Póvoa de Lanhoso) the trend remained.

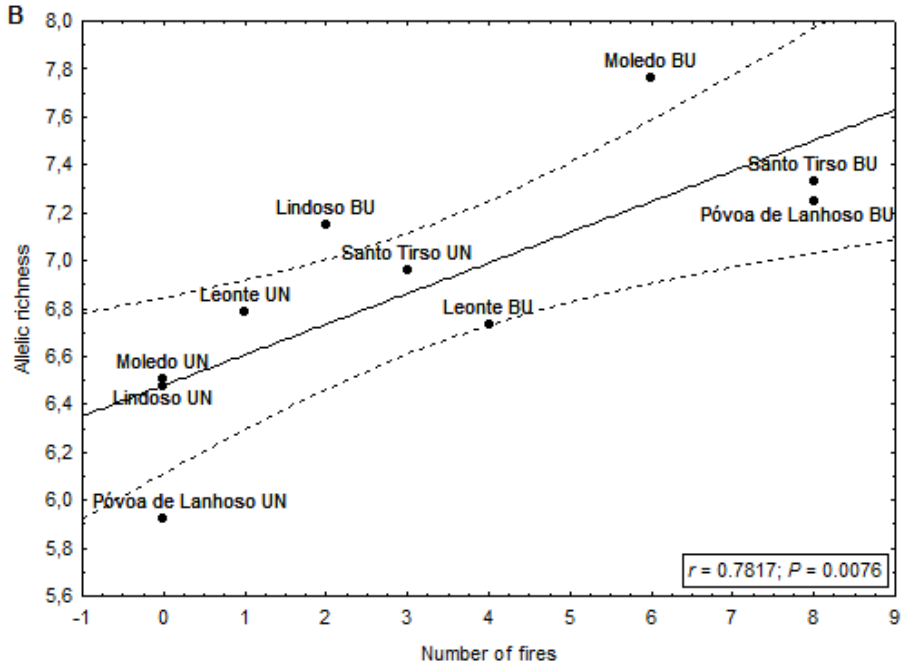
The relationships between genetic variables (N_A, P_A, H_E, F_{IS}) and the number of fires that occurred at each population (1975-2013), showed that all genetic metrics were positively correlated with the number of fires (see **Fig. 16**), with correlation coefficients ranging from 0.637 to 0.786 and probability values of approximately 0.01 (except for F_{IS} which was significant, $p < 0.05$). Thus, fire change the pattern of genetic diversity. Interestingly, the BU populations that have the lowest number of fires (Lindoso BU with 2 and Leonte BU with 4) appeared more proximate to the UN populations than other BU populations (see **Fig. 16-A, B, C**).

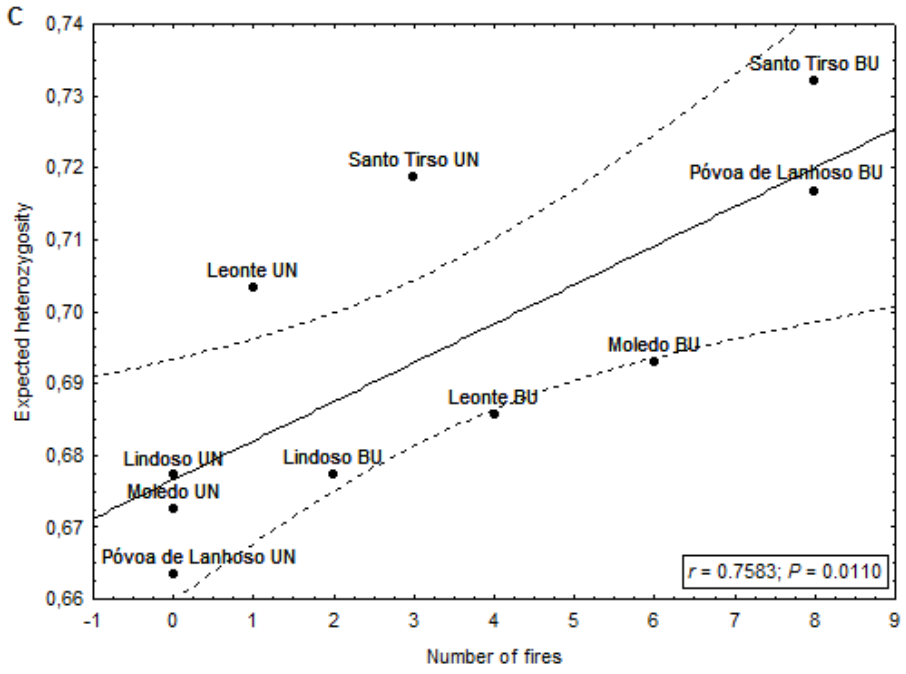
Table 3.6. Comparisons of genetic indices between populations of each pair. The *p* values obtained by 999 permutations. The same comparisons were performed for the pool of UN (unburnt) populations against BU (burnt) populations, and repeated having into account only the Gerês group. N_A - mean number of alleles; P_A - number of private alleles; H_E - expected heterozygosity.

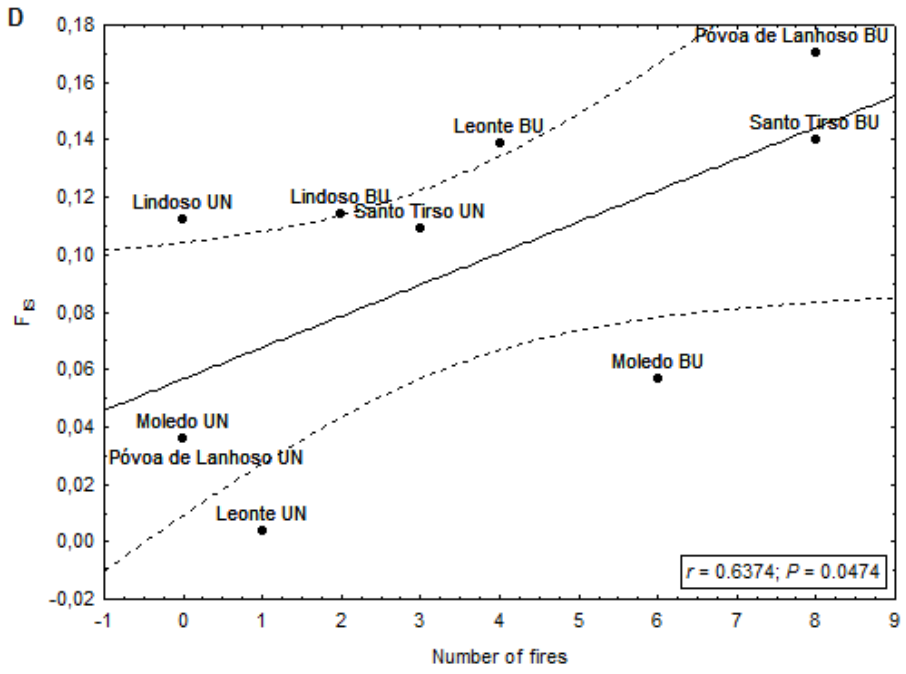
	N_A	*p*	P_A	*p*	H_E	*p*	F_{IS}	*p*
Leonte UN	7.125	0.376	8	0.376	0.703	0.757	0.004	**0.025**
Leonte BU	7.125		8		0.686		**0.139**	
Lindoso UN	6.750	**0.047**	9	**0.047**	0.677	0.440	**0.112**	0.492
Lindoso BU	7.250		13		0.677		0.114	
Santo Tirso UN	7.375	0.234	8	0.234	0.719	0.280	**0.109**	0.286
Santo Tirso BU	7.750		11		0.732		**0.140**	
Moledo UN	6.875	**0.001**	6	**0.001**	0.672	0.220	**0.036**	0.348
Moledo BU	8.625		20		0.693		**0.057**	
P. de Lanhoso UN	6.125	**0.005**	11	**0.005**	0.663	**0.030**	0.036	**0.022**
P. de Lanhoso BU	7.750		24		0.712		0.170	
UN populations	10.000	**$9x10^{-5}$**	2	**$9x10^{-5}$**	0.725	0.110		
BU populations	11.750		16		0.738			
Gerês UN	8.375	**$9x10^{-5}$**	2	**$9x10^{-5}$**	0.701	0.178		
Gerês BU	9.875		14		0.715			

Figure 3.7. Variation of (A) mean number of alleles; (B) allelic richness; (C) expected heterozygosity and (D) F_{IS}, with the number of fires that each population experienced from 1975-2013. The observed trend is an increase of all metrics with the number of fires. r – Linear correlation coefficient between variables.



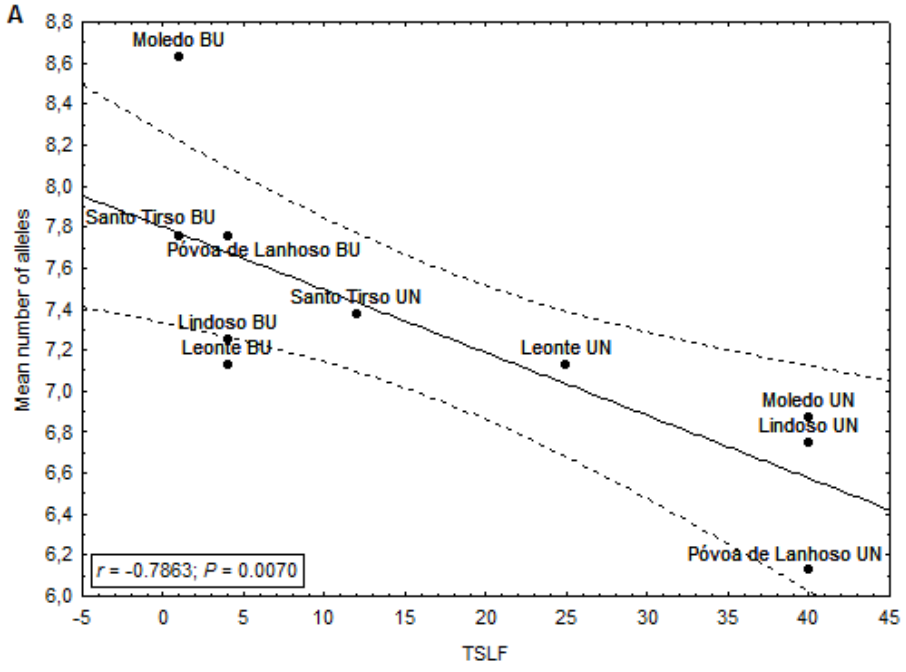

B
Allelic richness
Number of fires
Moledo BU
Santo Tirso BU
Póvoa de Lanhoso BU
Lindoso BU
Santo Tirso UN
Leonte UN
Leonte BU
Moledo UN
Lindoso UN
Póvoa de Lanhoso UN
r = 0.7817; P = 0.0076

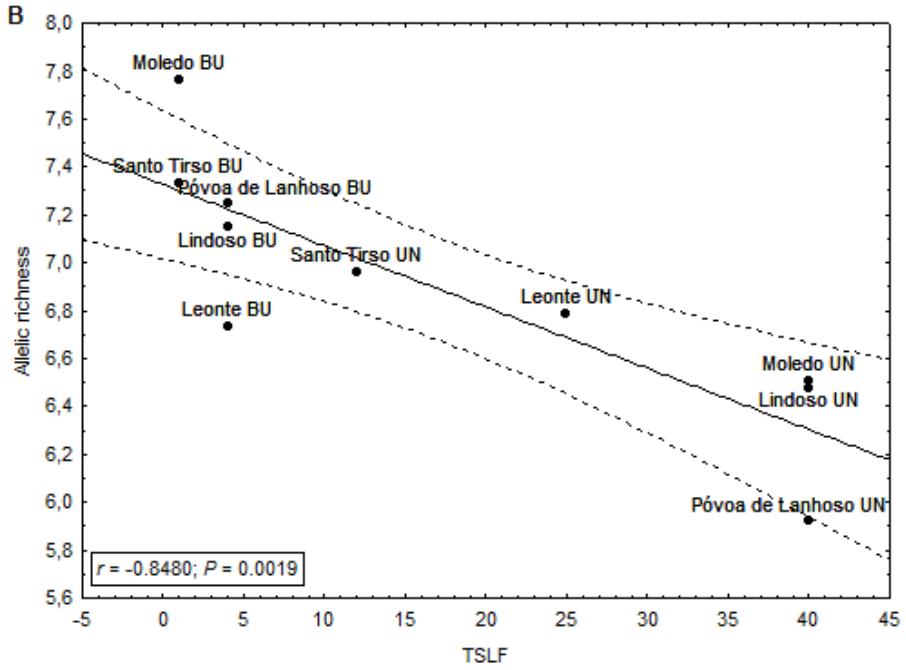

C
Expected heterozygosity
Number of fires
Santo Tirso BU
Santo Tirso UN
Póvoa de Lanhoso BU
Leonte UN
Moledo BU
Leonte BU
Lindoso UN
Lindoso BU
Moledo UN
Póvoa de Lanhoso UN
r = 0.7583; P = 0.0110

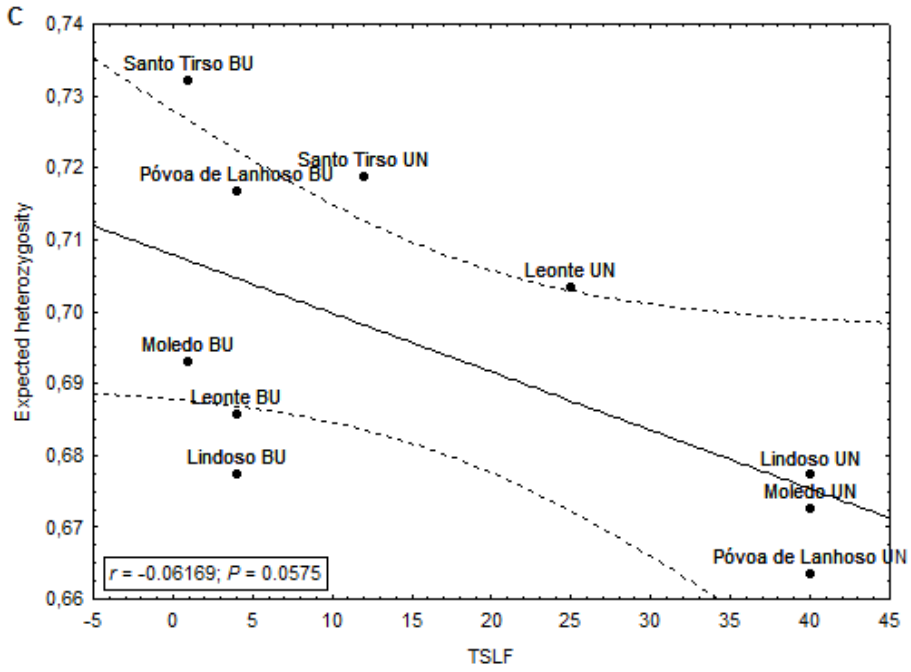


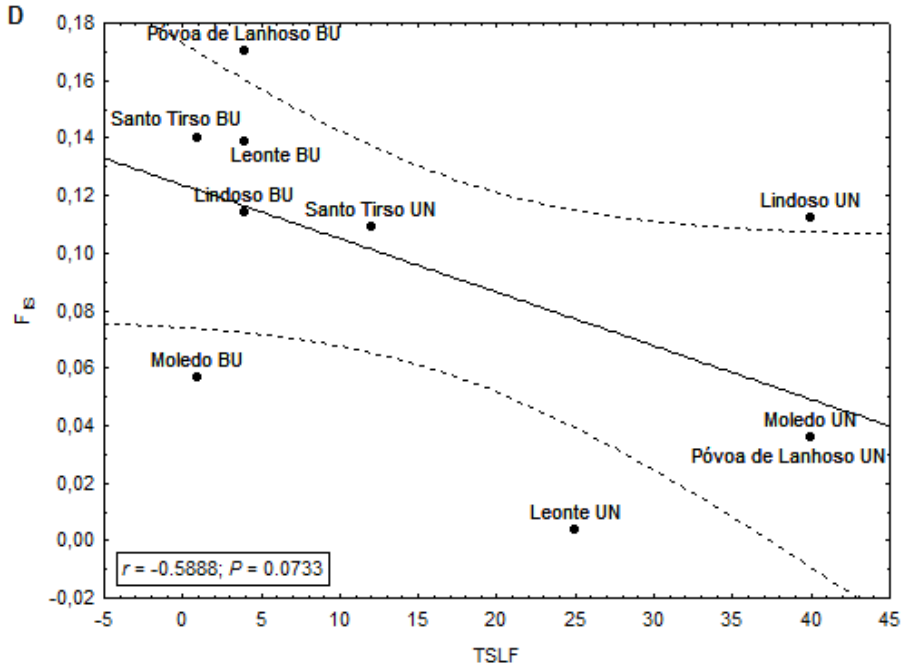
On the contrary, when the time since the last fire increased, all the diversity metrics decreased. Only the relationship between TSLF and the N_A and P_A were significant (see **Fig. 17-A; Fig. 17-B**), whereas between TSLF and H_E and F_{IS} was not significant (P = 0.057, **Fig. 17-C**; P = 0.073, **Fig. 17-D**).

Figure 3.8. Variation of (A) mean number of alleles; (B) allelic richness; (C) expected heterozygosity; and (D) F*IS* per population with the time since last fire (in years) from 1975-2013. The more recent the fires were, higher were all the genetic metrics. r – Linear correlation coefficient between variables.



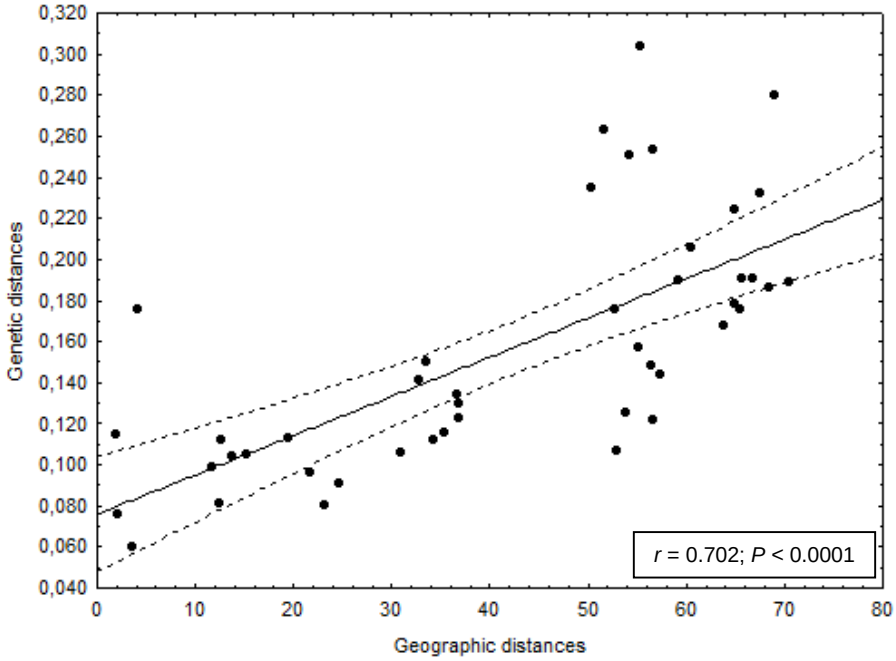

B
Allelic richness
TSLF
Moledo BU
Santo Tirso BU
Póvoa de Lanhoso BU
Lindoso BU
Santo Tirso UN
Leonte UN
Leonte BU
Moledo UN
Lindoso UN
Póvoa de Lanhoso UN
r = -0.8480; P = 0.0019


C
Expected heterozygosity
TSLF
Santo Tirso BU
Santo Tirso UN
Póvoa de Lanhoso BU
Leonte UN
Moledo BU
Leonte BU
Lindoso BU
Lindoso UN
Moledo UN
Póvoa de Lanhoso UN
r = -0.06169; P = 0.0575



The Mantel test showed a significant correlation between the genetic distance (Nei's D_{ST}, 1972) and the geographical distance (r = 0.702; p < 0.00004; 10,000 permutations; **Fig. 18**), meaning that the observed differences are most likely a result of spatial structure. In contrast, the Mantel test between the pairwise genetic distances and fire-history similarities was not significant (r = 0.06548; p < 0.32694; 10,000 permutations); the result was also not significant with a Partial Mantel test taking into account the geographic distances between populations (r = 0.180; p < 0.10515; 10,000 permutations). The Partial Mantel test showed that although there was not a significant correlation between genetic distances and fire-history while taking into account the geographic distances, fire actually disrupted the strong correlation between genetic and geographic distances by lowering the correlation coefficient (r). However, fire was not sufficiently important to shape the structure of *Podarcis* populations.

Figure 3.9. Relationship between pairwise Nei's D_{ST} genetic distance (1972) and geographic distances among all the sampled populations. Geographic distances are displayed in Km. r – Linear correlation coefficient between variables.



3.2.4. Recent demographic history

We wanted to test the hypothesis that BU population had experienced bottleneck imposed by the fire event. If the populations experienced a drastic reduction in population size it would leave a genetic signature that could be able to detect: a transient heterozygosity excess produced by a rapid loss of rare alleles (low frequency). All populations (UN and BU) were tested in order to be able to compare the results since UN populations are the control.

Under the Infinite Allele Model all populations exhibited heterozygosity excess detected by the Wilcoxon sign test, this is, a significant departure from mutation-drift equilibrium was found (**Table 10**). All the populations showed heterozygosity excess in more than half of the loci tested (≤ 5). The mode-shift test did not detect any evidence of a bottleneck (**Table 10**). The Two-phase Model is an intermediate model between Infinite Allele Model and Single Step Mutation Model, therefore we chose to tun it with 50% probability to have single step mutation (SMM) and 50% to have multiple steps (IAM). Here, there was a pattern of bottleneck in the UN populations, with exception of Moledo (**Table 10**). From the BU populations, only Santo Tirso BU showed signs of bottleneck (**Table 10**). When the Single Step Mutation Model was used, no signal of bottleneck was found. Apart from Lindoso UN in which 5 loci had heterozygosity excess, the remaining

populations had half or less (≤ 4) of loci with heterozygosity excess. The mode-shift test did not detect any evidence of a bottleneck (**Table 10**).

Nevertheless, the results from the test for allele frequency mode-shifts show that there is no distortion away from a normal L-shaped allele frequency distribution expected under mutation-drift equilibrium. According to this test, no population show sign of experiencing recent bottleneck.

These results are constrained by the mutation model model implemented since we do not know which model Podarcis microsatellites follows. We also have low number of markers, which may hamper the resolution to detect bottlenecks. For these reasons, we have contrasting results between mutation models, and also between different tests (Wilcoxon and Mode-shift tests). We cannot draw any conclusion from the results, this is, we cannot say for sure which populations experienced bottlenecks (if any).

The results of MIGRATE-N were inconsistent throughout the runs we did. Estimates of mutation-scaled migration (M) between pairs as well as the population mutation rate (Theta) varied in a way we could not draw any pattern. This may have arose by: i) the inherent error of the program (and priors are well estimated) and in which if population are similar in M and Theta values, with the software's error these values just fluctuate from one run to another; or ii) on the contrary, the variability of results are due to not well refined priors and we simply cannot interpret them. In either case, further analysis will have to be done in order to get reliable results.

Table 3.7. Results based on the over all loci heterozygosity excess test (Wilcoxon sign test: one tailed *p*) and allele frequency analysis (mode-shift) for all mutation models. H_E – Heterozygosity expected under Hardy-Weinberg quilibrium; H_{EQ} – Heterozygosity expected under mutation-drifr equilibrium; IAM – Infinite allele model; TPM – Two-Phase Model; SMM – Single Step Model. The TPM corresponds to 50% of multistep mutations and Variance = 12.

	H_O	IAM		TPM		SMM		Mode-shift
		H_{EQ}	Wilcoxon	H_{EQ}	Wilcoxon	H_{EQ}	Wilcoxon	
Leonte UN	0.721	0.626	**0.00195**	0.664	**0.00391**	0.702	0.52734	L-shaped
Leonte BU	0.703	0.639	**0.00977**	0.677	0.19141	0.722	0.87500	L-shaped
Lindoso UN	0.695	0.604	**0.00391**	0.642	**0.03711**	0.682	0.23047	L-shaped
Lindoso BU	0.698	0.654	**0.01953**	0.687	0.47266	0.726	0.98047	L-shaped
S. Tirso UN	0.737	0.651	**0.00391**	0.685	**0.01953**	0.722	0.47266	L-shaped
S. Tirso UN	0.751	0.683	**0.00195**	0.721	**0.02734**	0.763	0.98047	L-shaped
Moledo UN	0.690	0.633	**0.03711**	0.670	0.37109	0.711	0.87500	L-shaped
Moledo BU	0.709	0.661	**0.01367**	0.693	0.47266	0.726	0.90234	L-shaped
P. Lanhoso UN	0.680	0.618	**0.00391**	0.658	**0.03711**	0.706	0.96289	L-shaped
P. Lanhoso BU	0.735	0.687	**0.00391**	0.726	0.19141	0.766	0.99414	L-shaped

3.3. Morphologic component

The asymmetry index (AI) did not significantly deviate from normality within each population for any of the traits examined (Kolmogorov-Smirnov test, $p > 0.05$ in all cases), which allows to discard the presence of antisymmetry (i.e. one side is consistently larger than the other in a random manner among individuals).

Before proceeding to the analysis of asymmetry *per se*, it was done a test to check if the traits suffered from sexual dimorphism because it could inflate the presence of asymmetry. When all populations were pooled, we did not find sex differences in AI for the three traits examined (SCG: $t = -0.947$; $df = 188$; $p > 0.05$; FP: $t = -0.975$; $df = 196$; $p > 0.05$; SDL: $t = -0.049$; $df = 159$; $p > 0.05$). The t-test results for each location showed that Santo Tirso UN and BU and also Moledo BU showed significant differences in AI between sexes. Santo Tirso UN had differences statistically different between sexes in both FP and SDL ($t = 2.237$; $df = 18$; $p < 0.05$; $t = -2.803$; $df = 12$; $p < 0.05$, respectively), although the sex ration is unbalanced in this population (for FP there was 15 males for 5 females, while for SDL there was 11 males for 3 females). Santo Tirso BU showed differences in the SDL trait ($t = -2.193$; $df = 15$; $p < 0.05$), however this result could be due to the fact that the number of individuals between sexes are unbalanced (16 males – 1 female). Moledo BU also showed significant differences in SDL ($t = 3.025$; $df = 18$; $p < 0.01$) with a proportion of 14 males to 6 females. In summary, differences in sex relative to the AI were not the general pattern across all populations. For this reason, the analysis of asymmetry was done joining both sexes.

There was not a significant relationship between AI and the trait size; this means that the asymmetry is not higher for traits with higher number of scales (SCG, df =1, F = 0.991, $p = 0.320$; FP, df = 1, F = 1.557, $p = 0.214$; SDL, df = 1, F = 0.041, $p = 0.839$).

A two-way ANOVA was applied to assess which type of asymmetry (fluctuating or directional) was present in the dataset, taking into account the measurement error between counts. The results showed that the interaction between ‘Individual’ and ‘Side’ factors was significant for all traits and all populations ($p < 0.0001$), indicating fluctuating asymmetry for all traits and locations.

Table 3.8. Results from the two-way ANOVA. Dependent variables were log-transformed trait values (i.e. number of scales) and tested for each population and trait separately. 'Individual' was set as a random factor and 'Side' as a fixed effect. UN – unburnt; BU – burnt; *df* – degrees of freedom; *F* – F-statistics; *p* – *p* value.

Location	Condition	Trait	Individual			Side			Individual * Side		
			df	***F***	***p***	***df***	***F***	***p***	***df***	***F***	***p***
Leonte	UN	SCG	18	21.384	< 0.0001	1	0.010	0.922	18	4.531	< 0.0001
		FP	20	9.44	< 0.0001	1	0.00	0.986	20	104.580	< 0.0001
		SDL	16	38	< 0.0001	1	4	0.045	16	16	< 0.0001
Leonte	BU	SCG	17	4.819	< 0.0001	1	0.432	0.520	17	16,506	< 0.0001
		FP	19	4.89	< 0.0001	1	1.76	0.201	19	158.78	< 0.0001
		SDL	16	6.14	< 0.001	1	2.06	0.171	16	20.08	< 0.0001
Lindoso	UN	SCG	18	11.470	< 0.0001	1	0.047	0.831	18	8.770	< 0.0001
		FP	19	4.29	< 0.01	1	0.07	0.794	19	103.03	< 0.0001
		SDL	15	7.06	< 0.0001	1	1.33	0.267	15	15.60	< 0.0001
Lindoso	BU	SCG	16	8.099	< 0.0001	1	0.108	0.747	16	9.805	< 0.0001
		FP	14	2.5	0.048	1	0.74	0.405	14	117.74	< 0.0001
		SDL	15	17.88	< 0.0001	1	0.62	0.443	15	13.16	< 0.0001
Santo Tirso	UN	SCG	19	6.265	< 0.001	1	0.078	0.783	19	10.845	< 0.0001
		FP	19	19.89	< 0.0001	1	0.45	0.509	19	18.94	< 0.0001
		SDL	13	4.55	< 0.0001	1	0.10	0.757	13	11.48	< 0.0001
Santo Tirso	BU	SCG	20	10.687	< 0.0001	1	0.089	0.768	20	13.620	< 0.0001
		FP	20	3.06	< 0.01	1	0.03	0.866	20	61.01	< 0.0001
		SDL	16	15.57	< 0.0001	1	6.42	< 0.05	16	13.52	< 0.0001
Moledo	UN	SCG	15	2.725	< 0.05	1	1.954	0.182	15	6.121	< 0.0001
		FP	19	10.18	< 0.0001	1	0.02	0.880	19	35.76	< 0.0001
		SDL	9	4.28	< 0.05	1	0.27	0.613	9	69.43	< 0.0001
Moledo	BU	SCG	21	6.613	< 0.0001	1	0.356	0.557	21	10.503	< 0.0001
		FP	21	8.81	< 0.0001	1	0.92	0.348	21	70.61	< 0.0001
		SDL	19	5.62	< 0.001	1	0.02	0.896	19	18.24	< 0.0001
Póvoa de Lanhoso	UN	SCG	18	13.556	< 0.0001	1	2.084	0.166	18	4.887	< 0.0001
		FP	18	7.35	< 0.0001	1	0.78	0.390	18	15.34	< 0.0001
		SDL	14	12.3	< 0.0001	1	0.44	0.519	14	32.06	< 0.0001
Póvoa de Lanhoso	BU	SCG	18	12.341	< 0.0001	1	0.290	0.597	18	5.593	< 0.0001
		FP	19	12.42	< 0.0001	1	3.90	0.063	19	51.96	< 0.0001
		SDL	18	6.44	< 0.001	1	0.11	0.7743	18	117.92	< 0.0001

The four-way ANOVA on the log-transformed average of trait values (individual FA index) revealed that there were significant differences across the traits examined (F = 26.421; df = 2; $p < 0.0001$), where SCG had significantly higher FA than both FP (post-hoc Scheffé, p = 0.0000) and SDL (post-hoc Scheffé, p = 0.0000). The effect of condition type (UN vs. BU) was not significant (F = 3.685; df = 1; p = 0.055), but the interaction 'Condition*Trait' was significant (F= 3.258; df = 2; $p < 0.05$). This means that for SCG, lizards at BU populations were more asymmetric than lizards in UN populations, whereas no differences were found for the other two traits. (**Fig. 19**). Also, the interaction 'Location *Trait' was significant (F= 2.214; df = 8; $p < 0.05$), which suggests that there are differences in the degree of asymmetry in the traits examined between the pairs of populations of each location. This seems to be happening because SCG is not always the trait with the highest asymmetry values (**Fig. 20**). It was not found significant differences between sexes (**Table 12**).

Table 3.9. Results of the four-way ANOVA on the log-transformed average of trait values across the replicate counts. SS – sum of squares; *df* – degress of freedom; *F* – F-statistic; *p* – *p* value.

	SS	***df***	***F***	***p***
Condition	0,002373	1	3,6854	0,055474
Population (Condition)	0,000567	4	0,2203	0,927092
Sex	0,000010	1	0,0161	0,899228
Trait	0,034026	2	26,4205	**0,000000**
Population*Sex	0,001594	4	0,6189	0,649210
Condition*Sex	0,000906	1	1,4067	0,236185
Population*Trait	0,011403	8	2,2136	**0,025287**
Condition*Trait	0,004196	2	3,2581	**0,039296**
Sex*Trait	0,001402	2	1,0888	0,337442

Figure 3.10. Interaction between condition and trait. Both the supraciliar granules (SCG) and the femoral pores (FP) show higher level of asymmetry in populations of BU (burnt) condition. On contrast pattern is seen in the subdigital lamellae (SDL). Error bars represent 95% confidence intervals.

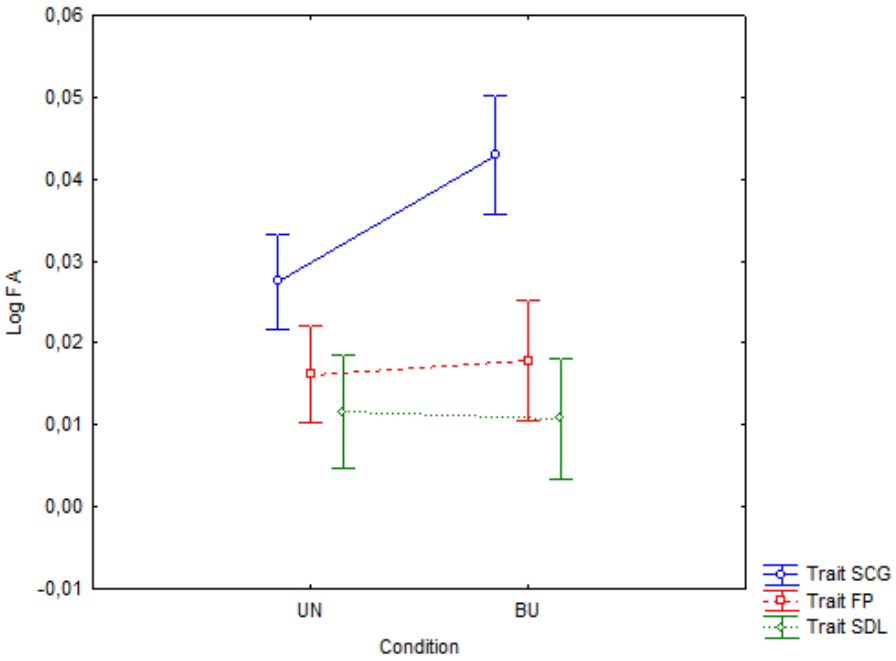
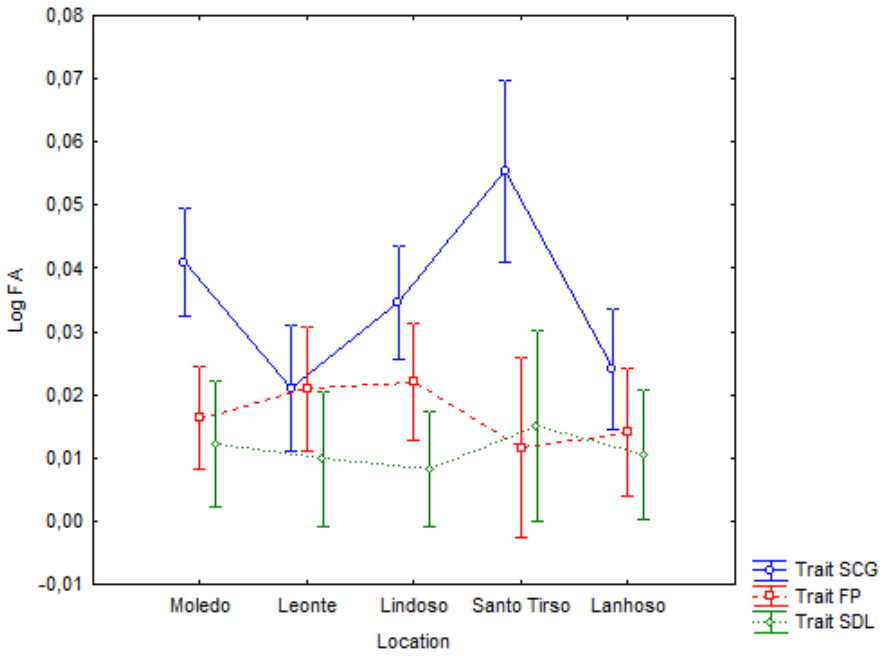


Figure 3.11. Mean degree of FA between populations (UN vs. BU) in each locations across all traits examined. The interaction between location and traits factor are suggested to be caused by the higher degree of FA in UN rather than in BU for both Moledo and Santo Tirso. Error bars represent 95% confidence intervals.



There were not found significant correlations between traits both in signed and unsigned FA (**Table 13**). Therefore, the degree of asymmetry between traits were not correlated, and it is not dependent of the body part in which the trait developed.

Table 3.10. **Correlations between pairs of traits in their signed and unsigned asymmetry index (R-L).**

	Signed FA			**Unsigned FA**		
	SCG R-L	**FP R-L**	**SDL R-L**	**SCG R-L**	**FP R-L**	**SDL R-L**
SCG R-L	-	$p = 0.689$	$p = 0.187$	-	$p = 0.189$	$p = 0.689$
FP R-L	$r = -0.033$	-	$p = 0.171$	$r = -0.108$	-	$p = 0.846$
SDL R-L	$r = -0.108$	$r = -0.112$	-	$r = -0.033$	$r = -0.016$	-

Given the previous results regarding the non-significant P value of the factor condition (UN vs. BU; **Table 12**) and the results that supported that Moledo and Santo Tirso were introducing bias with some contrasting patterns of fluctuating asymmetry of those of the remaining populations (see **Fig. 20**), the four-way ANOVA was repeated with the locations from the Gerês group (Leonte, Lindoso and Póvoa de Lanhoso). In fact, this approach would be more reliable since these populations are geographically closer, thus it can be assumed that they are under very similar conditions (which in turn was tested with the information from the dataloggers – see before).

There was not a significant relationship between AI and the trait size; this means that the asymmetry is not higher for traits with higher number of scales (SCG, df =1, F = 0.8415, $p = 0.361$; FP, df = 1, F = 0.0001, $p = 0.992$; SDL, df = 1, F = 0.0035, $p = 0.953$).

The four-way ANOVA based only on the degree FA of populations from the Gerês group revealed that the main factors ‘Condition’ (UN vs. BU) and ‘Trait’ were significant (**Table 14**). The interactions ‘Condition*Trait’ and ‘Sex*Trait’ were also found to be significant. The ‘Condition’ showed to be an important factor ($F = 4.855$; $df = 1$; $p = 0.028$) when discerning population relative to their degree of fluctuating asymmetry, in which BU populations showed to have higher degree of FA than the UN populations (post-hoc Scheffé, p = 0.0025). Regarding the ‘Trait’ factor ($F = 13.133$; $df = 2$; $p < 0.0001$), they were all statistically different from each other (post-hoc Scheffé, SCG and FP, P = 0.0001; SCG and SDL, $p = 0.0000$; FP and SDL, $p = 0.0449$) in which SCG>FP>SDL. There was a significant interaction between ‘Condition’ and ‘Trait’ ($F= 4.394$; $df = 2$; $p < 0.05$), which means that the degree of asymmetry in each trait is different regarding the condition of populations (**Fig. 21**). Post-hoc Sheffé tests indicated that FA was

significantly different between UN and BU population for the trait SCG (p = 0.0003) while there was no significant difference between UN and BU for remaining traits. Also, the interaction 'Sex*Trait' was significant (F= 4.548; df = 2; p < 0.05; **Table 14**). Nonetheless, the post-hoc Scheffé tests does not show any significant correlation between sexes in each trait.

Table 3.11. Results of the four-way ANOVA on the log-transformed average of trait values across the replicate counts for the Gerês group. SS – sum of squares; *df* – degress of freedom; *F* – F-statistic; *p* – *p* value.

	SS	*df*	*F*	*p*
Condition	0,002087	1	4,8553	**0,028347**
Population (Condition)	0,000013	2	0,0156	0,984491
Sex	0,000294	1	0,6833	0,409127
Trait	0,011289	2	13,1334	**0,000003**
Population*Sex	0,000194	2	0,2256	0,798180
Condition*Sex	0,000171	1	0,3984	0,528432
Population*Trait	0,002511	4	1,4607	0,214228
Condition*Trait	0,003777	2	4,3941	**0,013183**
Sex*Trait	0,003910	2	4,5481	**0,011353**

Figure 3.12. Interaction between condition and trait for the Gerês group ANOVA. Both the supraciliar granules (SCG) and the femoral pores (FP) show higher level of asymmetry in populations of BU (burnt) condition than of UN (unburnt) populations in the Gerês group. On contrast pattern is seen in the subdigital lamellae (SDL). Error bars represent 95%

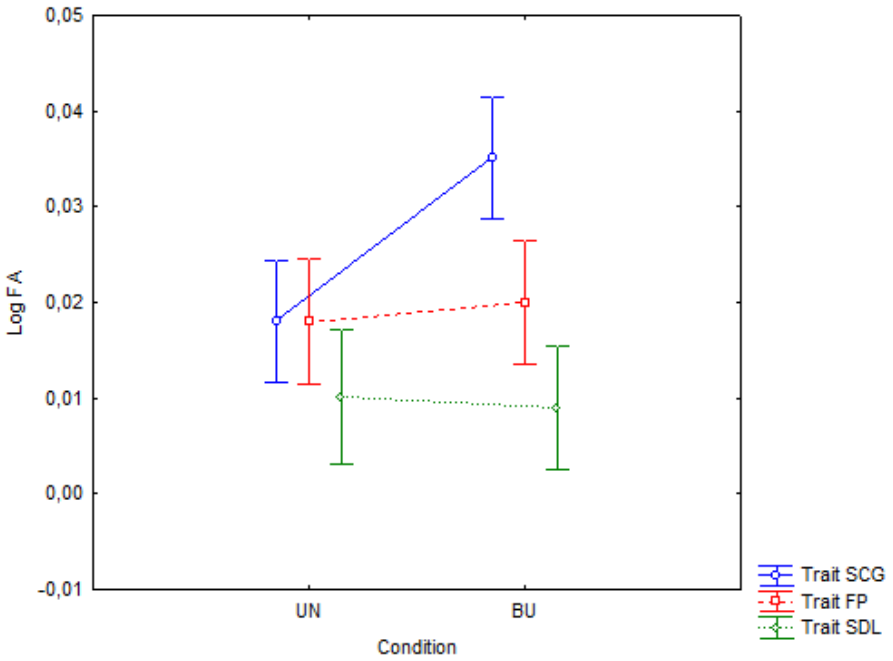
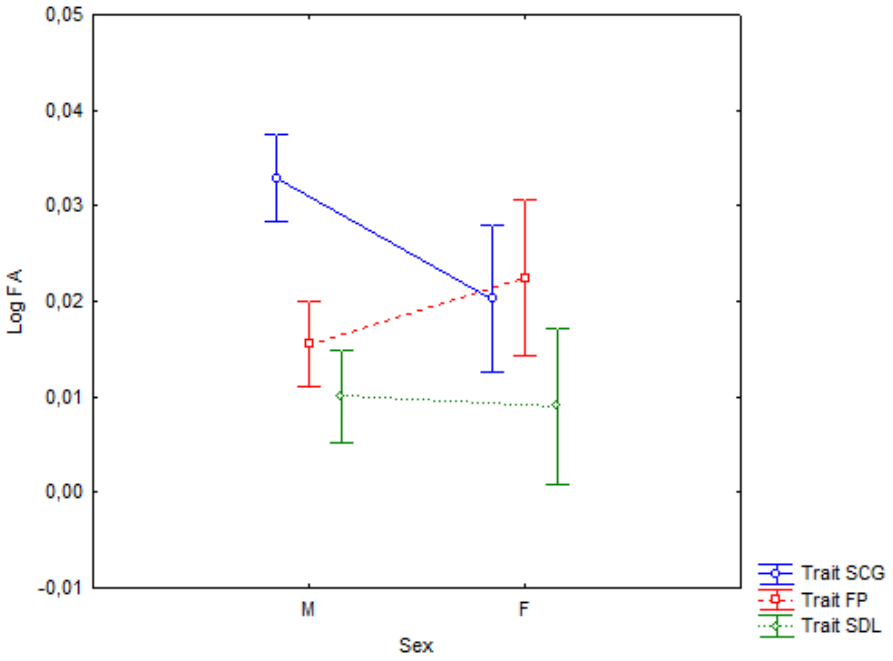


Figure 3.13. Interaction between sex and trait factors for the Gerês group ANOVA. Contrasting patterns is shown, in particular in supraciliar granules (SCG) and the femoral pores (FP) which are crossed-over: the males (M) showed higher degree in mean fluctuating asymmetry in SCG whereas in the FP the females (F) showed higher FA. The subdigital lamellae (SDL) showed similar resuts for both sexes. Error bars represent 95% confidence intervals.



There were not found significant correlations between traits both in signed and unsigned FA (**Table 15**). Therefore, the degree of asymmetry between traits were not correlated, and it is not dependent of the body part in which the trait developed.

Table 3.12. Correlations between pairs of traits in their signed and usigned asymmetry index (R-L) for the Gerês group.

	Signed FA			Unsigned FA		
	SCG R-L	**FP R-L**	**SDL R-L**	**SCG R-L**	**FP R-L**	**SDL R-L**
SCG R-L	-	p = 0.981	p = 0.181	-	p = 0.691	p = 0.675
FP R-L	r = 0.002	-	p = 0.711	r = 0.042	-	p = 0.279
SDL R-L	r = -0.141	r = -0.039	-	r = -0.045	r = -0.115	-

3.4. Genetic component and asymmetry

None (all populations sampled and the Gerês group) of the relationships between the individual FA index (for each trait separately and averaged across three traits) and the IMH were significant (only the FA averaged for the three traits of all populations sampled is shown; r = 0.0853, p = 0.2995; **Fig. 23**). The population FA index (for each trait separately and averaged across the three traits) was again not significantly correlated with any of the population genetic measures (N_A, A_R, H_E, and F_{IS}). Against our expectation that the level of asymmetry would be negatively correlated with the genetic diversity (according to the Heterozygosity Theory, Clarke, 1998), the genetic variability did not influenced the degree of asymmetry of populations (only the population asymmetry index averaged for the three traits is shown; r = 0.1748, p = 0.6292.

Figure 3.14. Linear regression between the individual multilocus heterozygosity (IMH) and the individual degree of asymmetry averaged across the three traits examined of all populations sampled. . r – Linear correlation coefficient between variables.

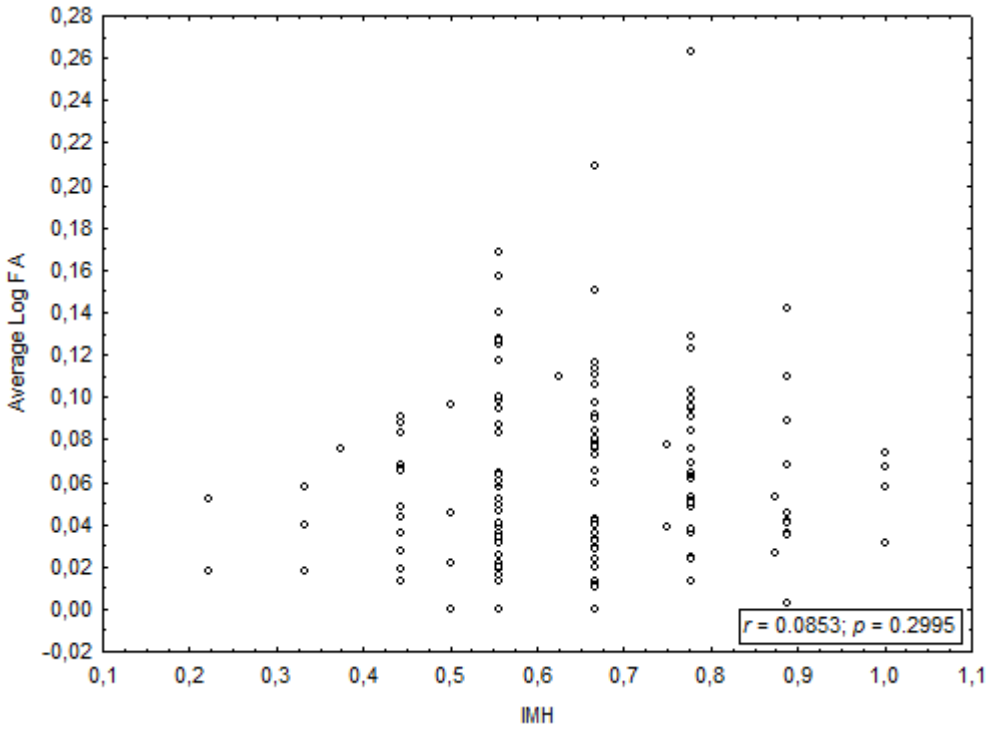
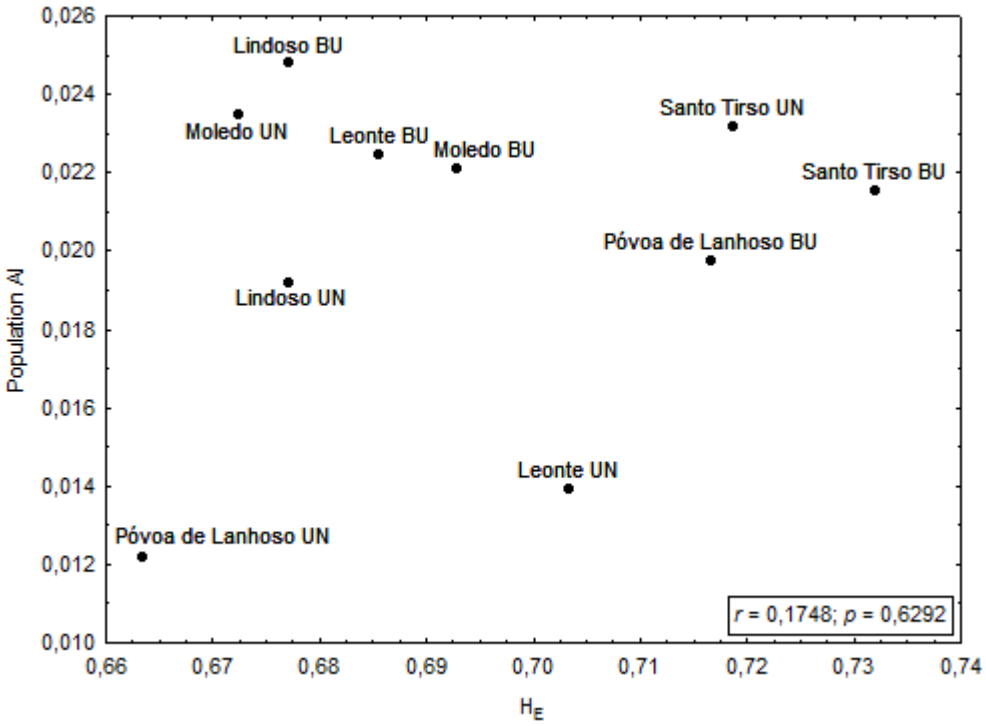


Figure 3.15. Linear regression between the population expected heterozygosity (H_E) and population asymmetry index averaged across the three traits examined for each populations sampled. r – Linear correlation coefficient between variables.



4. Discussion

Content

We used samples from 201 individual wall lizards from 10 geographically separated locations with different fire-histories to understand the role of repeated fires in shaping the genetic diversity and morphological traits in *Podarcis guadarramae* wall lizards in northwestern Portugal. Our study design (gradient of different fire-histories) gave the opportunity to perform tests to enlighten wich genetic patterns and processes can potentially be driven by the occurrence of wildfires, thus extend the knowledge in this research field that is currently lacking information (Steinitz *et al.*, 2012; Banks *et al.*, 2013). Additionally, we studied how fires can act on the development of morphological traits with ecological importance in order to understand the stability and viability of populations that experienced the repeated occurrence of natural disturbances.

4.1. Evaluating the impact of repeated fires on *Podarcis guadarramae* populations in northwestern Portugal

4.1.1. Environmental conditions

The first effect of fire is the consumption of vegetation. Post-fire environmental conditions are then altered due to structural changes in the habitat. Our expectations that the environmental conditions are harsher in burnt areas are confirmed by the increased amplitudes of temperature and humidity thus, showing that they receive more solar radiation due to vegetation simplification by fires. Burnt areas were hotter and drier than unburnt areas, which has important effects from the embryonic development to the distribution of animals.

4.1.2. Genetic impacts

The analyses of population genetics helped us to unravel how repeated fires impact the genetic diversity of *Podarcis guadarramae*. Our results clearly show that fires altered the genetic diversity of wall lizards. Our expectation was that fires would alter patterns of genetic diversity in burnt populations by decreasing it through successive bottlenecks and founder effects. These results have been found in populations of butterflies (Fauvelot *et al.*, 2006) and lizards (Schrey *et al.*, 2011a) and have been further attributed to bottlenecks in several taxa (e.g. mammals, Collevatti *et al.*, 2007; birds, Brown *et al.*,

2013; lizards, Ujvari *et al.*, 2008). As a consequence, burnt populations would become more differentiated from unburnt populations by means of genetic drift. Nevertheless, our results did not meet our expectations. Curiously, they showed precisely the opposite pattern – significantly increasing genetic diversity with number of fires, and conversely, a significant decreasing of genetic diversity with time since the last fire. This was valid not only for the overall correlation between fire and genetic diversity parameters but also, in general, to local pairwise comparisons. Regarding the BU-UN population pairs, we found a pattern of increased genetic diversity in BU except in Leonte. In Leonte, the unburnt population showed higher diversity than the burnt population. This site is located within the National Park Peneda-Gerês in which vegetation management is done by low intensity fires out summer period. This fact has been confirmed recently, since low intensity fires occurred in January-March 2015 in this population. The occurrence of (prescribed) low intensity fires perhaps can disrupt the general pattern of an increase of genetic diversity in the BU population with respect to the UN population. Also the burnt population of Lindoso is managed by prescribed fires. However, in both cases the likely underestimation of the number of fires did not affect the general pattern of increased genetic diversity with the number of fires. Moreover, the populations showed a significant, yet very shallow, fine-scale genetic differentiation, however it was not a product of fire-history but rather of isolation-by-distance. The incorporation of knowledge of both direct, in particular the demographic processes (Steinitz *et al.*, 2012), and indirect effects (shift in habitat structure) of fire is important to understand the underlying processes behind the patterns of genetic diversity found (Banks *et al.*, 2013).

Population recovery following major disturbances such as wildfires is likely a reflection of two non-mutually exclusive biological processes: i) abundance of survivors; and ii) post-fire recruitment (e.g. local reproduction or immigration) (Banks *et al.*, 2011; Steinitz *et al.*, 2012; Robinson *et al.*, 2013). Some studies have documented a short-term post-fire decline of fire-sensitive species (e.g. Santos *et al.*, 2015), although other studies of vertebrates have shown that fires rarely cause complete mortality and that residual populations remain *in situ* in burnt areas (e.g. Banks *et al.*, 2011; Sanz-Aguilar *et al.*, 2011; Suaréz *et al.*, 2012), specifically cliff- and rock-specialist species for which habitat is not destroyed by fire (Santos *et al.*, 2015). Biological processes such as mortality, survival and post-fire recolonization can affect the genetic diversity in contrasting ways (Banks *et al.*, 2013): reductions in heterozygosity and allelic richness are a result of population bottleneck (e.g. Brown *et al.*, 2013); increases in genetic diversity and reduced regional genetic differentiation as a result of a high immigration rate (e.g. Schrey *et al.*, 2011a; Pereoglou *et al.*, 2013); and the intermediate value of unchanged genetic diversity when mortality is low or is compensated by immigration (e.g.

Suaréz *et al.*, 2012). We suggest that in the case of *P. guadarramae* the pattern of higher genetic diversity (mean number of alleles, allelic richness, private alleles, expected heterozygosity) in burnt populations can be explained by a combination of both survival rate and immigration. The survival of individuals could be able to retain the pre-fire genetic diversity (Suaréz *et al.*, 2012; Banks *et al.*, 2015), and later, a migration of individuals from outside of fire boundary would bring new alleles hence, increasing diversity (Schrey *et al.*, 2011a; Banks *et al.*, 2015).

The likelihood of immediate survival of an individual during a fire will be influenced by the severity of the fire, its distance to potential refuges and behavioural mechanisms the organisms may use to avoid direct flames and heat (Friend, 1993; Whelan, 2002; Banks *et al.*, 2011). Refuges enhance immediate survival during a fire event but can also facilitate the post-fire persistence of individuals and populations within the burnt landscape (Robinson *et al.*, 2013). In the longer term while vegetation grows, refuges may contribute to re-establishment of populations in extensively burnt landscapes either as a source for population expansion from within the fire boundary and by facilitating the colonization of individuals from outside the fire boundary by providing resources in the short-term (food, shelter) or longer-term (resident habitat) (Banks *et al.*, 2011; Watson *et al.*, 2012; Robinson *et al.*, 2013). Although the spatial dynamics of species in such situations (i.e. expansion) is not entirely known, depending on the spatial isolation of refuges relative to the mobility of animals, spatial population structure within the burnt landscape may vary through time along a gradient from a series of disjunct isolated populations, to a metapopulation and to a patchy population linked by frequent movements (Templeton *et al.*, 2011; Driscoll *et al.*, 2012).

Our results suggest that refuges were preponderant both in the survival rate of *Podarcis* lizards as well as for colonizers. *Podarcis guadarramae* is a very saxicolous lizard and it is associated with rocky habitats (Sá-Sousa, 2000). Likewise, its preference for these habitats that naturally have a high number of crevices and burrows, where the lizard could refuge during the fire events, allows a considerable survival of animals. Moreover, due to the fact that fire opened the habitat, the unoccupied habitats increased and perhaps immigrant individuals took advantage of these new spaces and colonized them which increases the carrying capacity of the population. Nevertheless, we do not have a way to test this hypothesis since we do not have pre-fire genetic data nor movement data to corroborate this hypothesis. We have tested, however, if populations have experienced recent demographic bottlenecks (i.e. test if there was high mortality) by examining the genetic signature of this demographic process. Our results were not conclusive as to decide whether populations have experienced bottleneck. On one hand, under IAM all populations have been bottlenecked. On the other hand, under SMM no

population experienced a reduction in population size. However, the model-shift test of the distribution of allele frequencies also suggests that no population experienced bottlenecks. Genetic bottleneck tests require making assumptions about microsatellite evolution to generate expected distributions for test statistics (Cornuet and Luikart 1996; Garza and Williamson 2001). Results are highly sensitive to assumed parameter values, and incorrect assumptions about these parameter values can lead to an erroneous inference that a bottleneck occurred (Peery *et al.*, 2012). Under IAM, microsatellite loci have been shown to exhibit heterozygosity excess in stable populations (Luikart and Cornuet, 1998). Thus, likely our results under the IAM are not reliable. Bottleneck detection is further complicated by the fact that several factors including the timing and duration of the bottleneck, immigration and the amount of pre-bottleneck genetic diversity can influence and potentially obscure genetic signals of population declines (Cornuet and Luikart 1996; Garza and Williamson 2001; Peery *et al.*, 2012). Luikar and Cornuet (1998) suggest that in order to be statistically conservative one should use only the SMM when analysing microsatellite data to test for recent bottlenecks. Microsatellites have a complex mutation model in *Podarcis*, but more likely it does not follow a strict SMM model. Therefore, we cannot rely on neither results of bottlenecks.

This lizard has shown to cope very well with post-fire conditions since we have seen them throughout the burnt areas in National Park Peneda-Gerês (DF and XS, personal observations) which may explain the migration of individuals into burnt areas that brings new diversity (and increase allelic richness and heterozygosity). Banks *et al.* (2015) studied the capacity of refuges to buffer demographic and genetic processes in the moutain brushtail possum (*Trichosurus cunnunghami*) against drought and wildfires. Their results are in agreement with ours in which there was a higher genetic diversity in mesic unburnt vegetation (in our case, rocky outcrops which do not burn *per se*) after fire, as a result of high survival and immigration. They concluded that refuges were important for the resistance and stability of population both during drought conditions and post-fire persistence. In another study, Suaréz *et al.* (2012) saw that despite the demographic bottleneck imposed by a severe fire in the Gran Canaria chaffinch (*Fringilla teydea polatzeki*), the genetic diversity was retained. They pointed different factors than the former example: a possible combination of life history traits (juvenile dispersal and random mating thus, genetic diversity maintained), habitat characteristics (vegetation had fire adapted traits thus, rapid regeneration and recolonization by birds) and fire severity (heterogeneity of the fire event thus, birds moved to less severely affected areas) could have contributed to maintain genetic diversity. Schrey *et al.* (2011a) found the same result in one of the examined lizard species – increased diversity in burnt areas. They tested a Metapopulation Source/Sink model based on the habitat preferences of

each species, by examining the direction of gene flow (i.e. immigration into or out of preferred habitat). The burnt area indeed received migrant individuals of the open-habitat species which increased genetic diversity.

The observed general increment of genetic differentiation according to geographic distance (isolation by distance) and not to fire-history, suggested limited gene flow among all *P. guadarramae* populations which also figures a classical metapopulation scenario, especially when individuals show low dispersal ability like the case of lizards (Pannel and Charlesworth, 2000). At the UN-BU pairwise level, our results match the expected genetic outcome of increased adult immigration in spatially-structured populations, i.e. increased local genetic diversity but reduced regional genetic differentiation (Hansson *et al.*, 2003). Indeed, we found only low, but significant, population differentiation at almost all pairs, and in agreement, weak genetic structure between populations. Although fire did not overcome the effect of spatial structure, it did disrup the correlation of genetic and geographic differentiation by lowering the linear correlation coefficient and associated probability. The Metapopulation Source/Sink model can be applied to our species: fire stimulated gene flow and altered genetic diversity by causing the migration of individuals into the preferred habitat – open corridors and habitats that are provided by wildfire. The metapopulation theory provides a mechanism where recolonization of habitat patches counters the effects of local extinction (Hanski, 1998). One good example of this metapopulational dynamics and gene flow is the collared lizard (*Crotaphytus collaris*) case in the Missouri Ozarks in which fire increased the movement of individuals by promoting landscape permeability (Templeton *et al.*, 2001; Templeton *et al.*, 2011). Reptiles show a strong successional response to fire (Smith *et al.*, 2013) that is driven by changes to habitat structure and are a product of species life history (e.g. Santos *et al.*, 2015), thus as a general rule the open-habitat species are favoured by post-fire habitat conditions; then, while the ecological succession is recovering, forest-species increase its abundance whereas open-habitat species tend to decrease (e.g. Valentine *et al.*, 2012; Santos *et al.*, 2015). In our study areas (i.e. locations) these metapopulations could be spatially arranged according to the distribution of rocky and agricultural (walls) areas given its saxicolous ecology (Sá-Sousa, 2000). In unburnt populations these areas are in-between unsuitable habitat (e.g. high vegetation), therefore showing lower genetic diversity than burnt populations due to 'restricted' gene flow.

The impacts of fire may vary due to the heterogeneous characteristic of fire regimes (intensity, severity, etc.) and affect populations in different degrees due to its own processes and dynamics. Nevertheless, using replicate populations we were able to find a clear pattern of how fire can affect lizard populations.

4.1.3. Morphological impacts

The results obtained by examining the impacts of fire on the development stability of lizard population provide valuable information regarding the development of hatchlings under environmental stress. As hypothesized, increased levels of FA were observed in burnt populations compared to unburnt ones. Periods of rapid environmental change due to fires could have altered the individual's energy expenditure during development and thereby increase phenotypic variation in populations (Hoffmann and Parsons, 1997). Such increased variation was evident in deviations from optimal development in morphological traits, which was expressed as increased asymmetry in burnt population (Palmer and Strobeck, 1986). Under stressful conditions, the development of symmetrical traits might be more difficult to achieve. In our case, we tested temperature and humidity since these factors are known to cause major influence on lizards, therefore changes in these conditions can affect them in a negative way – developmental under stressful conditions (Angiletta *et al.*, 2010). Not exactly on the same research topic but still connected, Levy *et al.* (2015) recently suggested that previous population models underestimated the demographic impact of climate change by not taking into account micro-climate soil changes. In North American lizards, they showed that predicted fitness loss was about 2% by 2100, but it inflated to 35% when taking into account embryonic performance in response to hourly fluctuations in soil temperature. In conclusion they stated that accurate forecasts require detailed information of environmental conditions and thermal tolerances throughout the life cycle. We have earlier confirmed that burnt populations showed higher ranges of both temperature and relative humidity, thus higher environmental fluctuations. Based on our results of fluctuating asymmetry, we suggest that post-fire conditions on burnt populations imposed an environmental stress sufficiently strong to induce alterations in the correct developmental stability of individuals. Most probably, the most important factor is temperature since several measures differed significantly from UN to BU populations, whereas humidity only significantly differed in the range.

Reptile eggs and embryos are extremely sensitive to thermal and hydric regimes during incubation (Ackerman and Lott, 2004; Löwenborg *et al.*, 2011): they require appropriate levels of temperature, moisture and gas exchange for successful development (Shine and Thompson, 2006). Most species do not exhibit parental care, therefore the environment that eggs experience depends on the nest microhabitat. As a result, the thermal and hydric environments of nests can vary substantially over space and time (Warner *et al.*, 2010), which likely have important fitness consequences. Many studies show that temperature affect patterns of embryonic development and offspring

phenotypes in ways that can impact fitness. For example, Braña and Ji (2000) tested the influence of temperature on several aspects of *Podarcis muralis* hatchling phenotype likely affecting fitness by incubating the eggs under different temperature regimes. They observed that the thermal environment negatively affected size, mass and locomotor performance such as speed, in particular on higher temperatures. Another study that links the incubation temperature with phenotypic variability and reduced fitness but now on a skink (*Bassiana duperreyi;* Shine, 2004) tested the effect of seasonal shifts in incubation (nest) temperatures in which the regime only differed regarding the sequence of temperatures. The reptiles showed to be sensitive to seasonal shifts by modifying the time of hatching and developmental stability of embryos: they showed deformaties (generally in head and tail) as well as morphologic alterations (smaller body size) and reduced locomotor performance of hatchlings, generally in hatchlings from eggs exposed to falling temperatures. Downes and Shine (1999) went further and tested the influence of incubation temperature on the susceptibility of hatchlings of three lizard species to snakes. Their results show that apart from morphology and locomotor performance, also chemosensory responses to snake scent and ability to avoid a snake predator was observed.

The other condition, humidity, is less studied regarding to its effect on egg development. Higher humidity can increase hatchling size of some lizard species (e.g. Marco *et al.*, 2004; Warner *et al.*, 2012), and humidity fluctuations can also affect tail length and growth rate (Robbins and Warner, 2010). Nevertheless, humidity seems to have a negligible effect on embryogenesis and/or phenotypic traits of hatchlings in some species (e.g. Ji and Braña, 1999; Velo-Antón *et al.*, 2011). For example, Monasterio *et al.* (2013) tested both temperature and moisture on the reproductive traits of *Lacerta schreiberi* and saw that only temperature strongly and negatively affected the lizard. Moisture only a had a small positive effect on hatchling size constrained by low temperatures that the authors argue the it may provide some buffer against negative effects of temperature.

In terms of the effect of temperature and humidity on the FA during embryogenesis, there are very few studies which constrains further comparisons. On grass snakes (*Natrix natrix*), incubation of eggs under cooler conditions induced scale abnormalities and lower fitness due to smaller body and poor locomotor performance (Löwenborg *et al.*, 2011). The authors suggest that individuals with asymmetrical scales should rarely survive to adulthood in the wild due to disadvantages of disrupted embryogenesis, in particular in suboptimal nest-site choice by females. Conversely, a study performed in an Agamid lizard (*Calotes versicolor*) showed that at higher incubation temperatures several hatchling traits were negatively correlated (smaller body

size, higher incidence of deformed embryos, etc) as well as an increased FA in the eye diameter (Ji *et al.*, 2002).

Although there is a general pattern of higher FA in burnt *P. guadarrame* populations, the degree of FA varied across traits. Such differences in stability both between populations and traits have led to the widespread use of developmental stability analysis as a technique for identifying and characterizing populations subject to systematic stress (Leary and Allendorf, 1989; Parsons, 1992). The SCG exhibited higher FA degree compared to FP and SDL across all populations, this means that developmental stability could be trait specific. We hypothesised that the FA degree could be also associated to trait functional significance, in which traits with a functional importance show lower FA (Palmer and Strobeck, 1986). Indeed, our results show that FP as well as SDL, both have known important functions (e.g. femoral pores are linked with reproductive behaviour, Lopéz *et al.*, 2002; subdigital lamellae have been related to climbing capacity and habitat use, Glossip and Losos, 1997). However, SCG have no evident known functionality. Since both FP and SDL have important biological functions, their development could be under stricter control, as selection for developmental canalization is expected to be stronger in traits of functional significance (Leamy and Klingenberg, 2005). Another reason is that there could have had selection against more asymmetric individuals in those traits, hence, we did not sampled them. Additionally, these traits are important not only due to their biological function *per se* but they reflect the limb length which can have locomotor consequences of the individual and its survival. One possible consequence of higher degree of asymmetry could be lower locomotor performance and its relation with speed to avoid predators, as showed in previous examples (e.g. Downes and Shine, 1999). These results match those of Lazić *et al.* (2013), although they have examined how pollution affects the developmental stability of *Podarcis muralis* populations. Accordingly, they observed the same patterns as ours, polluted populations (i.e. burnt) where more asymmetric than unpolluted ones (i.e. unburnt). These means that at least in *Podarcis*, this set of three traits are informative as to whether they are under environmental stress or not.

Besides environmental stress, genetic stress due to loss of diversity could also influence the developmental stability of populations. As the development of both sides of a bilaterally symmetrical trait is presumably under the influence of a single set of genes (Palmer and Strobeck, 1986; Markow, 1995), differences in asymmetry between the two sides of the body represent variation of environmental origin (Palmer and Strobeck, 1986). However, the tendency to develop in a stable path and to exhibit asymmetry may be influenced by genetic factors (Palmer and Strobeck, 1986). The Heterozygosity Theory (Clarke, 1998) argues that higher levels of genetic diversity (i.e. heterozygosity)

has a buffering role by increasing biochemical diversity and thus enabling stable development pathway in changing environments. However, a meta-analysis of 41 studies testing the relationship between FA and heterozygosity showed that only a very small proportion of the variance in developmental instability, as estimated by FA, was explained by heterozygosity levels (Vøllestad *et al.*, 1999). Likewise, the levels of FA were not significantly correlated with the heterozygosity both at the individual and population level. In a study with both *Podarcis guadarramae* (as *P. hispanica* in this work) and *P. bocagei*, their results suggested that FA was a function of isolation in island populations with, for example, higher FA in remote islands (isolated, probably with less variability) than that close to mainlaind (isolated by less time, probably with more variability). Our populations, though, are not isolated as seen by the low levels of differentiation (Bancila *et al*., 2010). An implicit assumption in various studies testing the relationship between asymmetry and heterozygosity is that the latter represents the genome-wide levels of genetic stress. However, when empirically tested, studies focus on a selected subsample of loci, and in most cases the degree to which these actually represent genome-wide phenomena remains unknown. Furthermore, microsatellites are neutral markers which means that probably they don't capture any adaptive processe that may affect the developmental stability of populations. Maybe using selective markers would help to uncover such relationship of genetic stress and developmental stability.

5. Conclusions

We have demonstrated that the repeated fire regime affects *Podarcis guadarramae* populations at both genetic and morphological level. On one hand, the genetic diversity was positively correlated with the number of fires and negatively correlated with time since last fire. On the other hand, although fire influenced the genetic structure of populations, it did not overcome the influence of spatial structure. The increased genetic diversity of burnt populations compared to unburnt ones may be a result of two non-exclusive biological processes: survival rate and immigration. During the fire event, wall lizard are most likely within refuges which enhance immediate survival and short-time persistence in the burnt area, retaining the majority of genetic diversity. As fire opens the habitat there will be more unoccupied habitat which will facilitate colonization from the outside of the fire boundary and increase the carrying capacity of the population. This is particularly important to this species since its preferred habitat are rocky areas, which obviously remain unaltered after fire. Hence, the immigration of individuals would bring new alleles, therefore increasing the genetic diversity. Population differentiation was low, although significant, and was positively correlated with geographic distances (isolation by distance). Accordingly, we found only weak genetic structure between populations. All these aspects point to a metapopulation dynamic. Fire stimulated gene flow and altered genetic diversity by causing migration of individuals into the preferred habitat of *Podarcis guadarramae* – open habitats that are provided by wildfire. Although fire did not rule the structure of populations, it disrupted the correlation between genetic differentiation and geographic distances by inducing changes in the genetic diversity.

The post-fire environmental conditions imposed an environmental stress (higher temperature ranges) that likely affected the stable development of populations from burnt areas. *Podarcis* populations in these areas showed a higher degree of fluctuating asymmetry. Fluctuating asymmetry is proposed to be a biological monitor of environmental stress that allows to detect early stressed populations. Asymmetries have been related with lower fitness in other studies (small sizes, lower locomotor performance; see references in previous section). One possible consequence of higher degree of asymmetry in *P. guadarramae* could be lower locomotor performance and its relation with speed to avoid predators. However, the degree of fluctuating asymmetry varied among traits according to their functional significance. Thus, traits with important biological functions would be submitted to selection, this fact reducing the degree of asymmetry at population level due to its impact on individual fitness.

Despite the idea that genetic diversity have a buffering role, we did not observed any correlation between the degree of asymmetry and heterozygosity at both individual and population level. This may be cause by using neutral markers that do not figure adaptive genes.

To our knowledge, this type of inter-disciplinary analysis was never done before. The genetic studies only addressed some aspects such as how fire impact on the genetic diversity, differentiation and structure (e.g. Fauvelot *et al.*, 2006; Ujvari *et al.*, 2008; Suaréz *et al.*, 2012; Schrey *et al.*, 2012b; Brown *et al.*, 2013; Banks *et al.*, 2015) or social behaviour and life-history traits (e.g. Banks *et al.*, 2012; Pierson *et al.*, 2013; Pereoglou *et al.*, 2013; Smith *et al.*, 2014). Then again, there is no study regarding the impact of fire on developmental stability on lizards. In summary, the repeated fire regime in northwestern Portugal affected positively the genetic diversity and negatively the morphological traits of the lizard *P. guadarramae*. The impact of fires is not negative in all aspects as firstly hypothesized. Instead it gave new insights of demographic processes behind post-fire responses of lizards. However, the impact on morphological traits has shown that populations on burnt areas are stressed and perhaps less viable (e.g. lower fitness). Given the results of this study, I hope it encourages further work in this research field in order to continue to fill the knowledge gap that exists in every taxonomic group. On one hand it is important to fill this gap because we still know very little about the impacts of fires to natural populations at these levels. On the other hand, forecasted scenarios of increasing fire activity further ensures that we need to know how this major disturbance affects organisms at all levels in order to be able to predict responses.

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7. Supplementary material

Table S1. Primer quantity and concentrations and respective primer tails with fluorescent label in each PCR reaction. All forward primers were diluted in 1:10 ultra-pure water.

Multiplex	Locus	Qt. of primer (v_F=50 µL)	Concentration (µM)	Primer tail
Mix C	Ph17	1.0	0.20	NED
	Ph21	0.5	0.10	VIC
	Ph30	0.7	0.14	FAM
	Ph38	0.5	0.11	PET
	Ph50	0.35	0.07	VIC
Mix E	Ph43	0.7	0.30	FAM
	Ph70	1.5	0.20	PET
	Ph81	0.7	0.14	FAM
	Ph128	1.0	0.14	VIC

Table S2. Jaccard similarity matrix of fire-history between pairwise populations.

	Leonte UN	Leonte BU	Lindoso UN	Lindoso BU	S. Tirso UN	S. Tirso BU	Moledo UN	Moledo BU	P. Lanhoso UN	P. Lanhoso BU
Leonte UN	0.000000	28.571429	66.666667	40.000000	44.444444	36.363636	66.666667	40.000000	66.666667	36.363636
Leonte BU	28.571429	0.000000	33.333333	50.000000	33.333333	28.571429	33.333333	30.769231	33.333333	42.857143
Lindoso UN	66.666667	33.333333	0.000000	50.000000	25.000000	20.000000	100.00000	22.222222	100.000000	20.000000
Lindoso BU	40.000000	50.000000	50.000000	0.000000	20.000000	16.666667	50.000000	18.181818	50.000000	33.333333
S. Tirso UN	44.444444	33.333333	25.000000	20.000000	0.000000	50.000000	25.000000	53.333333	25.000000	50.000000
S. Tirso BU	36.363636	28.571429	20.000000	16.666667	50.000000	0.000000	20.000000	58.823529	20.000000	55.555556
Moledo UN	66.666667	33.333333	100.000000	50.000000	25.000000	20.000000	0.000000	22.222222	100.000000	20.000000
Moledo BU	40.000000	30.769231	22.222222	18.181818	53.3333333	58.8235294	22.222222	0.000000	22.222222	58.823529
P. Lanhoso UN	66.666667	33.333333	100.000000	50.000000	25.000000	20.000000	100.00000	22.222222	0.000000	20.000000
P. Lanhoso BU	36.363636	42.857143	20.000000	33.333333	50.000000	55.555556	20.000000	58.823529	20.000000	0.000000

Table S3. Presence of null alleles (NA) and stuttering of the genotyped loci in each population. Locus Ph17 was eliminated.

	Ph17	Ph21	Ph30	Ph38	Ph50	Ph43	Ph70	Ph81	Ph128	
Leonte UN	NA									
Leonte BU			NA			NA			NA	
Lindoso UN			NA						NA; Stutter	
Lindoso BU					NA				NA; Stutter	
S. Tirso UN	NA		NA							
S. Tirso BU	NA					NA				
Moledo UN	NA									
Moledo BU	NA									
P. Lanhoso UN	NA		NA	NA					NA; Stutter	
P. Lanhoso BU	NA									

Table S4. Pairwise Nei's genetic distance (D_{ST}; Nei, 1972).

	Leonte UN	Leonte BU	Lindoso UN	Lindoso BU	S. Tirso UN	S. Tirso BU	Moledo UN	Moledo BU	P. Lanhoso UN	P. Lanhoso BU
Leonte UN	0.000	0.060	0.099	0.112	0.122	0.144	0.206	0.148	0.080	0.091
Leonte BU	0.060	0.000	0.104	0.105	0.107	0.125	0.190	0.157	0.113	0.096
Lindoso UN	0.099	0.104	0.000	0.115	0.168	0.178	0.251	0.235	0.106	0.116
Lindoso BU	0.112	0.105	0.115	0.000	0.191	0.191	0.304	0.263	0.141	0.123
S. Tirso UN	0.122	0.107	0.168	0.191	0.000	0.076	0.186	0.176	0.150	0.134
S. Tirso BU	0.144	0.125	0.178	0.191	0.076	0.000	0.189	0.232	0.112	0.130
Moledo UN	0.206	0.190	0.251	0.304	0.186	0.189	0.000	0.176	0.253	0.280
Moledo BU	0.148	0.157	0.235	0.263	0.176	0.232	0.176	0.000	0.176	0.224
P. Lanhoso UN	0.080	0.113	0.106	0.141	0.112	0.130	0.253	0.176	0.000	0.081
P. Lanhoso BU	0.091	0.096	0.116	0.123	0.134	0.130	0.280	0.224	0.081	0.000